

Hydrogen Sulfide Stimulates Ischemic Vascular Remodeling Through Nitric Oxide Synthase and Nitrite Reduction Activity Regulating Hypoxia-Inducible Factor-1 α and Vascular Endothelial Growth Factor-Dependent Angiogenesis

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Background—Hydrogen sulfide (H₂S) therapy is recognized as a modulator of vascular function during tissue ischemia with the notion of potential interactions of nitric oxide (NO) metabolism. However, little is known about specific biochemical mechanisms or the importance of H₂S activation of NO metabolism during ischemic tissue vascular remodeling. The goal of this study was to determine the effect of H₂S on NO metabolism during chronic tissue ischemia and subsequent effects on ischemic vascular remodeling responses.

Methods and Results—The unilateral, permanent femoral artery ligation model of hind-limb ischemia was performed in C57BL/6J wild-type and endothelial NO synthase-knockout mice to evaluate exogenous H₂S effects on NO bioavailability and ischemic revascularization. We found that H₂S selectively restored chronic ischemic tissue function and viability by enhancing NO production involving both endothelial NO synthase and nitrite reduction mechanisms. Importantly, H₂S increased ischemic tissue xanthine oxidase activity, hind-limb blood flow, and angiogenesis, which were blunted by the xanthine oxidase inhibitor febuxostat. H₂S treatment increased ischemic tissue and endothelial cell hypoxia-inducible factor-1 α expression and activity and vascular endothelial growth factor protein expression and function in a NO-dependent manner that was required for ischemic vascular remodeling.

Conclusions—These data demonstrate that H₂S differentially regulates NO metabolism during chronic tissue ischemia, highlighting novel biochemical pathways to increase NO bioavailability for ischemic vascular remodeling. (*J Am Heart Assoc.* 2012;1:e004093 doi: 10.1161/JAHA.112.004093)

Key Words: angiogenesis • ischemia • nitric oxide • vascular endothelial growth factor • xanthine oxidase

Hydrogen sulfide (H₂S) is a colorless, flammable, water-soluble gas with a pungent odor of rotten eggs,^{1,2} which is produced from various sources in mammalian tissues, where it is generated during cysteine/homocysteine metabolism.³ H₂S is produced predominantly by tissue-specific enzymes,^{4,5} including cystathionine- β -synthase, cystathionine- γ -lyase, and 3-mercaptosulfurtransferase. Similar to other gaseous mediators (eg, nitric oxide [NO] and

carbon monoxide), H₂S plays an important role in cardiovascular, neuronal, and endocrine systems, including ischemic and inflammatory processes.^{6–10}

H₂S has been reported to be beneficial for cardiovascular dysfunctional states involving different responses. For example, H₂S promotes vascular smooth muscle relaxation and induces vasodilation of isolated blood vessels by opening adenosine triphosphate-sensitive potassium (K-ATP) channel.¹¹ It has been reported that H₂S potentiates antioxidant defenses via enhanced production of glutathione.¹² H₂S also promotes tissue cytoprotection, as illustrated by inhibition of myocyte apoptosis during myocardial ischemia-reperfusion.¹³ More recently, it has been proposed that H₂S–NO interactions could occur, yet clear insight into this relationship is lacking. Conflicting reports have shown that H₂S can (1) directly inhibit NO synthase (NOS) enzyme activity, (2) confer protection against experimental cardiac arrest in an endothelial NOS (eNOS)-dependent manner, (3) interact with NO to form unique reactive species involved in cardiovascular function, and (4) inhibit phosphodiesterase-5 activity

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Received July 11, 2012; accepted August 14, 2012.

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potentiating NOS activity.^{14–18} Unfortunately, no mechanistic information exists about how H₂S affects NO metabolism and bioavailability during ischemic tissue vascular remodeling.

Here, we identify novel H₂S–NO crosstalk pathways, whereby H₂S stimulates NOS expression as well as xanthine oxidoreductase (XO)–mediated nitrite anion reduction to NO, which subsequently regulates ischemic vascular remodeling responses. We also report measurement of therapeutic plasma and tissue free H₂S levels and downstream molecular pathways that regulate these responses. These data demonstrate that H₂S serves an important role in maintaining tissue NO bioavailability during chronic ischemia.

Methods

Chemicals and Reagents

General chemicals and tissue culture reagents were obtained from Sigma Chemicals. Anhydrous sodium sulfide was purchased from Alfa-Aesar Inc. Anti-Ki67 antibody was obtained from Abcam Inc (Cambridge, MA, USA). Anti-CD31 antibody was obtained from BD Biosciences (San Jose, CA, USA). Vectashield plus DAPI was obtained from Vector Laboratories. All secondary fluorophore-labeled antibodies were obtained from Jackson ImmunoResearch Inc (West Grove, PA, USA). The murine MS-1 endothelial cell line was obtained from ATCC.

Animals and Experimental Procedures

Animals

C57BL/6J, B6.129P2-*Nos3^{tm1Unc}/J* (eNOS-knockout) and Db/Db diabetic male mice were purchased from Jackson Labs and used in this study. Mice were maintained at the Association for Assessment and Accreditation of Laboratory Animal Care International–accredited Louisiana State University Health Science Center–Shreveport animal resource facility and were maintained in accordance with the National Research Council's *Guide for Care and Use of Laboratory Animals*. All animal studies were approved by the institutional animal care and use committee (protocol P-08-041) and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Mouse Hind-Limb Ischemia Model and Treatment Routes

Hind-limb ischemia was induced in 12- to 16-week-old C57BL/6J wild-type and eNOS-knockout male mice and in 36-week-old Db/Db diabetic male mice, as we have reported previously.^{19,20} After anesthesia with ketamine/xylazine (100 mg/kg or 83 mg/kg) injection, ligation of the left

femoral artery was performed. Mice were randomly assigned to different experimental groups by one investigator and were treated and evaluated by a second blinded investigator. Sodium sulfide with or without inhibitors was administered in the retro-orbital capillary plexus.

Laser Doppler Blood Flow Measurements

Laser Doppler blood flows were measured with a Vasamedics Laserflo BPM2 device in the gastrocnemius before ligation, after ligation, and at the indicated days after ligation, as we have reported previously.^{19,20}

Vascular Density and Cell Proliferation Measurements

Vascular density (anti-CD31) and cell proliferation (anti-Ki67) measurements were performed as we have reported previously.¹⁹ Briefly, the gastrocnemius muscles from ischemic and nonischemic hind limbs were removed, dissected, and embedded in Optimal Cutting Temperature freezing medium and were stained with anti-CD31, anti-Ki67, and DAPI for immunofluorescent imaging. Simple PCI software version 6.0 (Compix Inc, Sewickly, PA) was used to quantitatively measure the surface area of CD31, Ki67, and DAPI staining.

Vascular Endothelial Growth Factor Enzyme-Linked Immunosorbent Assay

Ischemic and nonischemic tissue isolated from PBS control or H₂S-treated wild-type/eNOS-knockout mice was harvested at various time points, and vascular endothelial growth factor (VEGF) expression was measured by enzyme-linked immunosorbent assay (ELISA) with the R&D VEGF Quantikine ELISA kit, as per the manufacturer's instructions.

Xanthine Oxidase Activity ELISA Assay

Ischemic and nonischemic tissue from control or H₂S-treated mice was harvested at day 10. Xanthine oxidase activity was measured with an ELISA kit from Cayman Chemicals, as per the manufacturer's instructions.

Cyclic Guanosine Monophosphate ELISA Assay

Ischemic and nonischemic tissue from control or H₂S-treated mice was harvested at day 10, and cyclic guanosine monophosphate (cGMP) level was measured with a cGMP assay from Sigma, as per the manufacturer's instructions.

Measurement of Plasma and Tissue Free H₂S Levels

H₂S was measured with monobromobimane (MBB) as we have reported.²¹ Thirty microliters of plasma or tissue supernatant was reacted with 70 μ L of Tris-HCl (100 mmol/L, pH 9.5, 0.1 mmol/L DTPA) and 50 μ L of MBB solution (10 mmol/L) at 1% oxygen in a hypoxic chamber at room temperature. Sulfide-bimane (SDB) reaction product was analyzed with a

Shimadzu Prominence Ultra Fast high-performance liquid chromatography system. Specimen concentration was calculated by comparison to SDB sulfide standards.

Measurement of NO Generation

A Sievers 280i NO analyzer was used to measure H₂S-mediated NO production as we have reported.¹⁹ Muscle tissues were homogenized in Hank's balanced salt solution, and 200 μg total protein was injected into the reaction chamber for measurements of nitric oxides (NOx). Endothelial cells were exposed to 4 hours of either hypoxia (1% O₂) or normoxia (21% O₂) and were harvested, and 1 × 10⁶ cells in Hank's balanced salt solution were added into the reaction chamber. Sodium sulfide subsequently was injected into the chamber, and NO production was measured over time. Separate experiments with hypoxic endothelial cells plus the inhibitors 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO), paraformaldehyde, sulfanilamide, N-Ethylmaleimide (NEM), N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester (L-NAME), and febuxostat also were performed. Cell free protein experiments with recombinant XO were added to the reaction chamber at concentrations of 0.01, 0.005, and 0.0025 units, and 50 μmol/L sodium sulfide was then injected into the chamber. NO production was

determined by integrating the emission signal over time calibrated to a standard curve of nitrite (0.1, 0.5, 1, 10, and 100 μmol/L) reduced to NO in sodium iodide/glacial acetic acid.

Statistical Analysis

Data were reported as mean ± standard error of the mean for all groups. Statistical analysis was performed with Mann-Whitney or Kruskal-Wallis analysis of variance with Dunn's multiple-comparison tests in GraphPad Prism software (GraphPad software, San Diego, CA).

Results

Plasma and Tissue H₂S Levels Required for Ischemic Tissue Cytoprotection

H₂S has been reported to confer cytoprotection during various forms of tissue ischemia, yet little information is available about specific therapeutic concentrations of free H₂S necessary for protective effects. Therefore, we examined a range of sodium sulfide doses (H₂S donor), from 0.1 to 1 mg/kg, administered via retro-orbital injection twice daily,

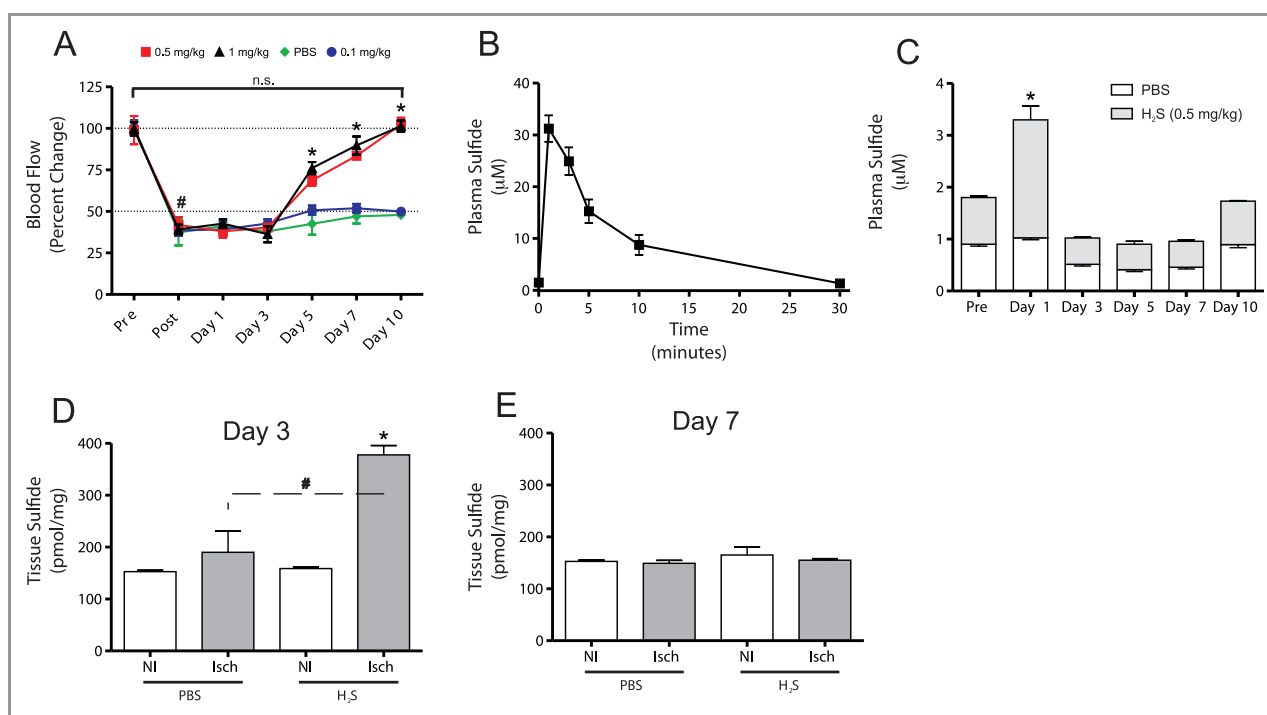


Figure 1. H₂S restores blood flow in permanent femoral artery ligation-induced hind-limb ischemia. A, Ischemic hind-limb blood flow changes with increasing concentrations of sodium sulfide. B, Free plasma H₂S levels after a single bolus injection of 0.5 mg/kg sodium sulfide. C, Steady-state free plasma levels of H₂S during the course of therapy. D, Tissue free H₂S levels in ischemic (Isch) and nonischemic (NI) tissues at day 3 after ligation. E, Tissue free H₂S levels in ischemic and nonischemic tissues at day 7 after ligation. n=12 animals per experimental cohort, *P<0.05 compared to control or nonischemic limb data; #P<0.05 ischemic limb comparison between PBS and H₂S, or before and after ligation.

to better understand therapeutic H₂S levels necessary for ischemic tissue cytoprotection. Sodium sulfide doses of 0.5 mg/kg (12.8 μmol/kg per day) and 1 mg/kg (25.6 μmol/kg per day) significantly restored ischemic hind-limb blood flow by day 10 after ligation (Figure 1A). Conversely, 0.1 mg/kg (2.6 μmol/kg per day) did not significantly alter ischemic hind-limb blood flow compared to PBS control. Thus, the therapeutic dose of 0.5 mg/kg was used for the rest of the study. Figure 1B illustrates the pharmacokinetic profile of plasma H₂S levels after a single injection of 0.5 mg/kg sodium sulfide, revealing a quick rise of plasma H₂S levels to ≈32 μmol/L within 1 minute of injection, which falls rapidly back to baseline levels (≈600 nmol/L) within 30 minutes. We next examined steady-state plasma free H₂S levels taken 2 hours after the first morning dose of 0.5 mg/kg sodium sulfide, as shown in Figure 1C. Steady-state free plasma H₂S is elevated within 1 day of beginning therapy and then decreases between days 3 and 7 and returns to baseline levels by day 10. We next measured tissue free H₂S levels at days 3 and 7 in nonischemic and ischemic tissues from sodium sulfide- and PBS-treated mice. Ischemic tissue H₂S levels from sodium sulfide-treated animals were elevated significantly above those of PBS-treated controls at day 3 (Figure 1D). Importantly, tissue H₂S levels were increased only in ischemic tissues, which suggests preferential bioavailability of H₂S in ischemic tissue. Figure 1E shows that by day 7, tissue H₂S levels had returned to baseline. Figure 2A through 2D show the representative photomicrographs of angiogenic index in non-ischemic and ischemic tissues with or without H₂S treatment. Figure 2E and 2F represent the H₂S dose-dependent stimulation of ischemic tissue angiogenesis (CD31/DAPI) and cell proliferation activity (Ki67/DAPI), respectively. These data reveal that H₂S plays major role in restoration of ischemic hind-limb blood flow or ischemic vascular remodeling and also indicate rapid and selective augmentation of free H₂S bioavailability during chronic tissue ischemia.

H₂S Restores Ischemic Tissue Blood Flow in a NO-Dependent Manner

Previous reports suggested that H₂S confers cardioprotective effects involving NO metabolism.^{15,17} Therefore, we administered an NO scavenger, cPTIO (1 mg/kg), to determine whether NO is involved in mediating ischemic tissue reperfusion during H₂S therapy. Figure 3A shows that cPTIO significantly blunts the effects of H₂S therapy on ischemic tissue blood flow in wild-type mice, demonstrating a key role for NO. Next, experiments were performed with eNOS-knockout mice to determine whether H₂S therapy mediates its effects on NO metabolism through this enzyme. Figure 3B shows that H₂S therapy significantly restored ischemic hind-limb blood flow in eNOS^{-/-} mice to 75% of preligation levels compared to PBS therapy. Plasma steady-state H₂S levels

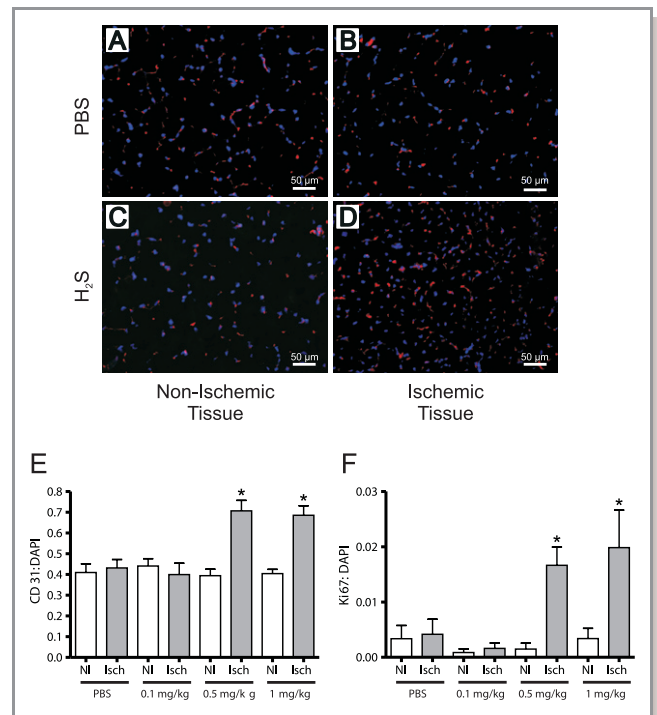


Figure 2. H₂S enhances ischemic vascular density and cellular proliferation. A and B, Tissue sections from PBS-treated animals both nonischemic and ischemic, respectively, stained for DAPI (blue) and CD31 (red) at day 10. C and D, Tissue sections from 0.5 mg/kg sodium sulfide-treated animals both nonischemic and ischemic, respectively, stained for DAPI (blue) and CD31 (red) at day 10. E, Graphical representation of the CD31:DAPI ratio (red:blue) demonstrating vascular density in different gastrocnemius tissues from sodium sulfide-treated mice. F, Graphical representation of Ki67:DAPI ratio (green:blue) demonstrating proliferation in different gastrocnemius tissues from sodium sulfide-treated mice. NI indicates nonischemic; Isch, ischemic. n=12 animals per cohort, *P<0.05 compared to PBS ischemic tissue data.

were measured in eNOS^{-/-} mice receiving different treatments: PBS therapy with 0.55±0.06 μmol/L versus sodium sulfide therapy with 0.89±0.06 μmol/L (P<0.05) plasma free H₂S. We next examined whether other NOS activity (inducible NOS [iNOS] and neuronal [nNOS]) could be involved in H₂S augmentation of eNOS^{-/-} ischemic hind-limb blood flow by using the NOS blocker L-NAME (5 mg/kg). L-NAME did not affect H₂S-dependent restoration of eNOS-deficient ischemic hind-limb reperfusion, suggesting that other NOS activity is not involved in this response (Figure 3C). Experiments were then performed in eNOS^{-/-} animals with cPTIO plus H₂S, which completely abrogated H₂S-mediated increased ischemic hind-limb blood flow (Figure 3D). cPTIO completely blocks H₂S-mediated angiogenesis and cell proliferation responses in ischemic tissues in wild-type animals (Figure 4A and 4B, respectively). Importantly, Figure 4C and 4D show that cPTIO but not L-NAME significantly prevents H₂S-mediated increases in the CD31:DAPI and Ki67:DAPI

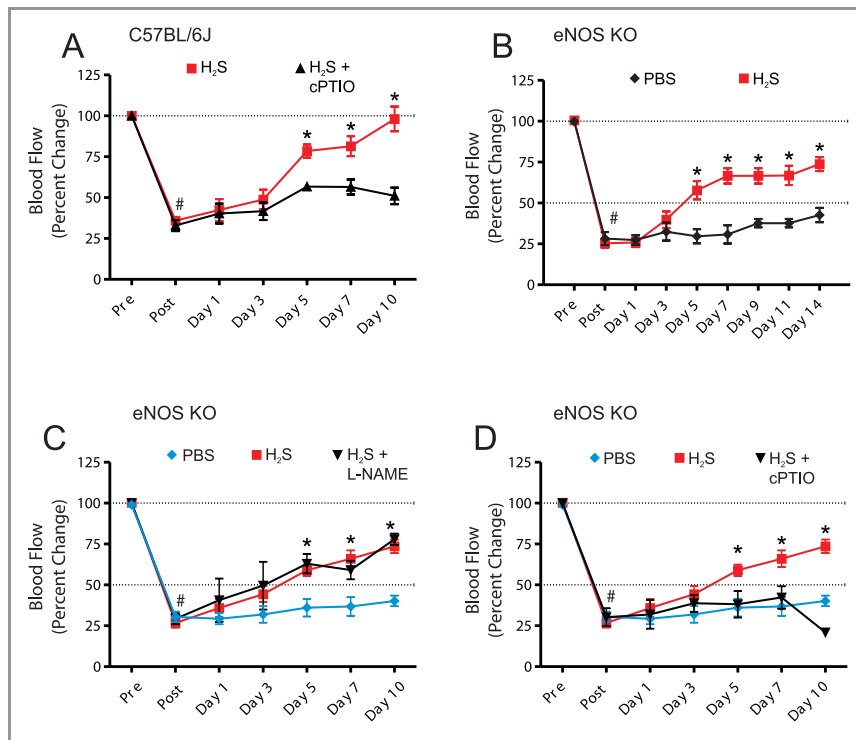


Figure 3. Role of NO and eNOS in H₂S restoration of ischemic limb blood flow. A, Blood flow from wild-type animals after sodium sulfide (0.5 mg/kg) therapy with and without cPTIO (1 mg/kg) to scavenge NO. B, Blood flow from eNOS-knockout (eNOS KO) animals with sodium sulfide therapy (0.5 mg/kg). C, Blood flow results from sodium sulfide-treated eNOS^{-/-} animals plus 5 mg/kg L-NAME (NOS inhibitor). D, Blood flow results from sodium sulfide-treated eNOS-knockout animals plus 1 mg/kg cPTIO (NO scavenger). n=12, #P<0.05 compared from before to after ligation, *P<0.05 compared to either H₂S+cPTIO or PBS controls.

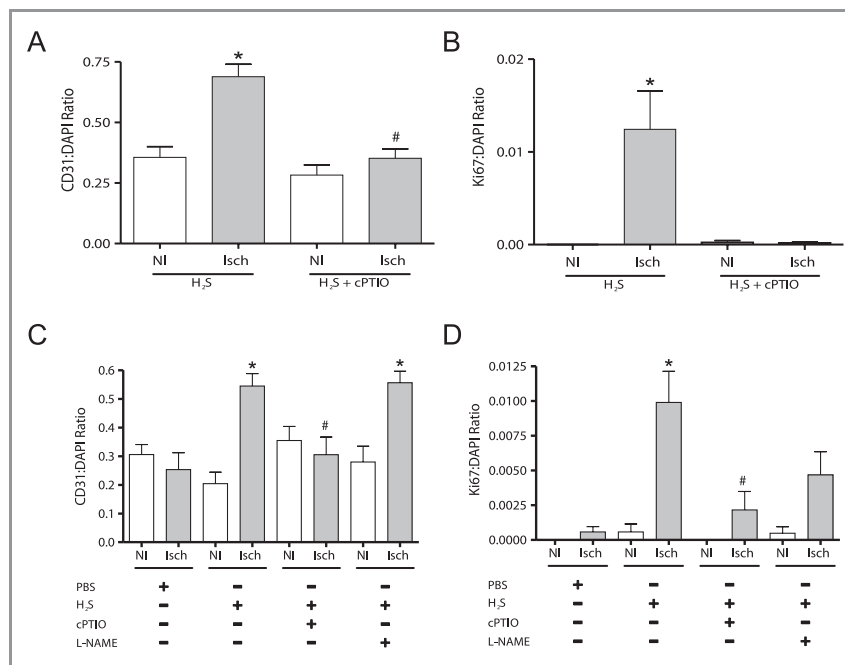


Figure 4. H₂S enhances vascular density in eNOS^{-/-} mice. A and B, Graphical representation of the CD31:DAPI and Ki67:DAPI ratios demonstrating vascular density and proliferation responses in wild-type mice treated with cPTIO. C and D, Vascular density and proliferation data of gastrocnemius tissues from eNOS^{-/-} mice under H₂S, cPTIO, and L-NAME treatments. NI indicates nonischemic; Isch, ischemic. n=12 animals per cohort, *P<0.05 compared to PBS ischemic tissue data, #P<0.05 H₂S versus H₂S+inhibitor treatments.

ratios of ischemic tissue in eNOS^{-/-} animals, confirming the importance of alternative NO generation for H₂S-mediated ischemic angiogenesis.

Increased NO Production and cGMP Expression by H₂S Therapy in Ischemic Tissue

Experiments then were performed to measure NO metabolites in plasma and tissue during H₂S therapy. Figure 5A shows that H₂S increases plasma nitrite levels at day 3 in wild-type mice and further increases plasma nitrite, nitrosothiol, and NO-heme levels by day 10 of therapy. Figure 5B reveals that total NOx levels (a combination of nitrite, nitrosothiol, and nitrosoheme) were selectively and significantly increased in ischemic tissues from wild-type mice at day 10 alone. Similar NO metabolite measurements were performed in eNOS^{-/-} mice, with Figure 5C illustrating that H₂S therapy did not significantly increase plasma NO metabolites at either time point. Interestingly, tissue total NOx levels were also selectively increased by H₂S therapy in ischemic tissues from eNOS^{-/-} mice (Figure 5D), although tissue NOx levels in eNOS^{-/-} mice were smaller than those of wild-type mice. These findings were confirmed independently with cGMP ELISA, which is indicative of NO generation. Figure 5E shows that H₂S therapy selec-

tively increases ischemic tissue cGMP production in both wild-type and eNOS^{-/-} mice. Figure 6 reports Western blot analysis of NOS total protein expression and eNOS phosphorylation at different times in tissues from wild-type and eNOS^{-/-} mice. These blots reveal that total eNOS expression and phosphorylation was increased at day 3 but not at day 10 in wild-type mice. Moreover, an increase in total iNOS expression was observed in ischemic tissues at day 3 and decreased by day 10 in both mouse genotypes, whereas nNOS expression was moderately increased in nonischemic and ischemic tissue from wild-type and eNOS^{-/-} mice. Together, Western blot data, NO metabolite measurements, and ischemic limb blood flow data implicate a role for NOS activity during H₂S therapy; however, NOS blockade in eNOS^{-/-} mice did not attenuate the effects of H₂S on ischemic vascular remodeling, suggesting other mechanisms of NO production.

H₂S Stimulates Nitrite Reduction to NO Under Hypoxia

We next examined whether H₂S could increase NO bioavailability through NOS-independent mechanisms. Endothelial cells were subjected to normoxic or hypoxic conditions, placed into a NO-chemiluminescent reaction vessel, and

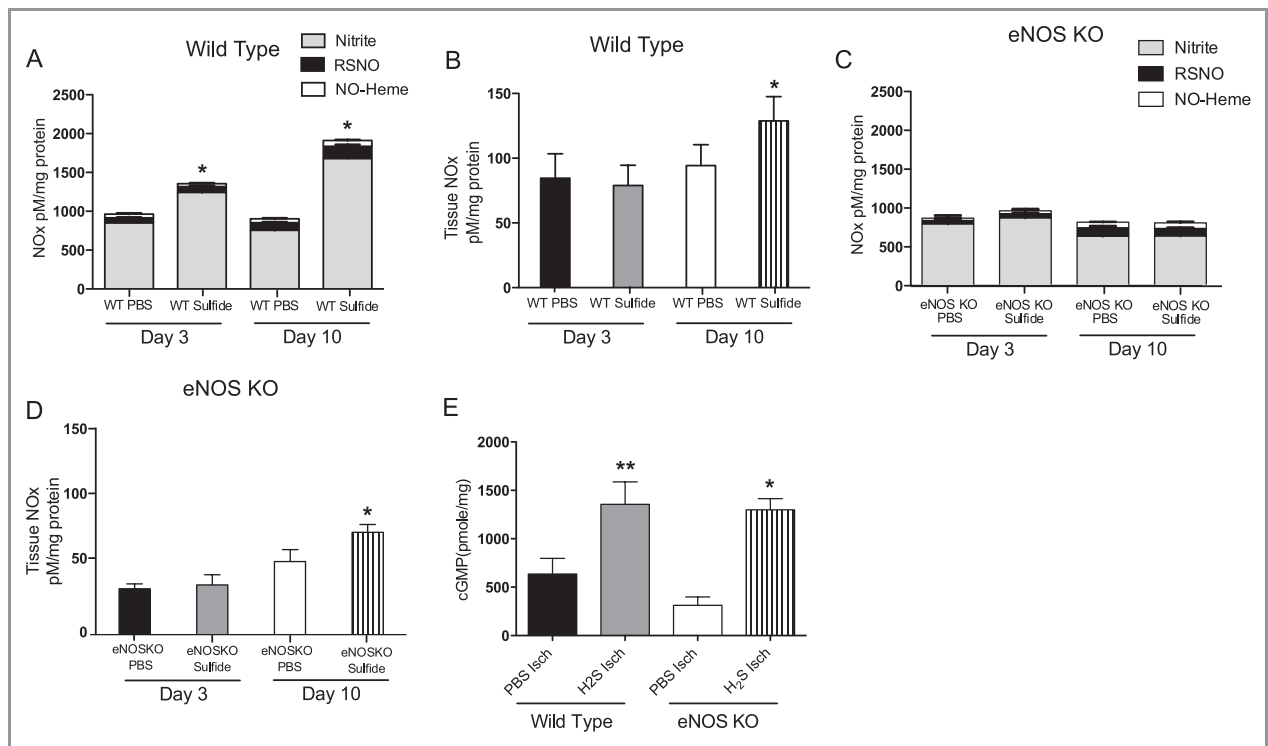


Figure 5. H₂S effects on plasma and tissue NO formation and bioavailability. A and B, H₂S-induced plasma and tissue NOx levels, respectively, in wild-type mice at day 3 and day 10 after ligation. C and D, H₂S-mediated NOx levels in plasma and ischemic tissues of eNOS^{-/-} mice at day 3 and day 10 after ligation. E, H₂S-mediated cGMP levels in ischemic and nonischemic tissues from wild-type and eNOS^{-/-} mice at day 10 after ligation. RSNO indicates nitrosothiol; WT, wild type; KO, knockout; and Isch, ischemic. n=5 animals per cohort. **P*<0.05, ***P*<0.001 compared to PBS control data, #*P*<0.05 compared to eNOS^{-/-} PBS ischemic tissue data.

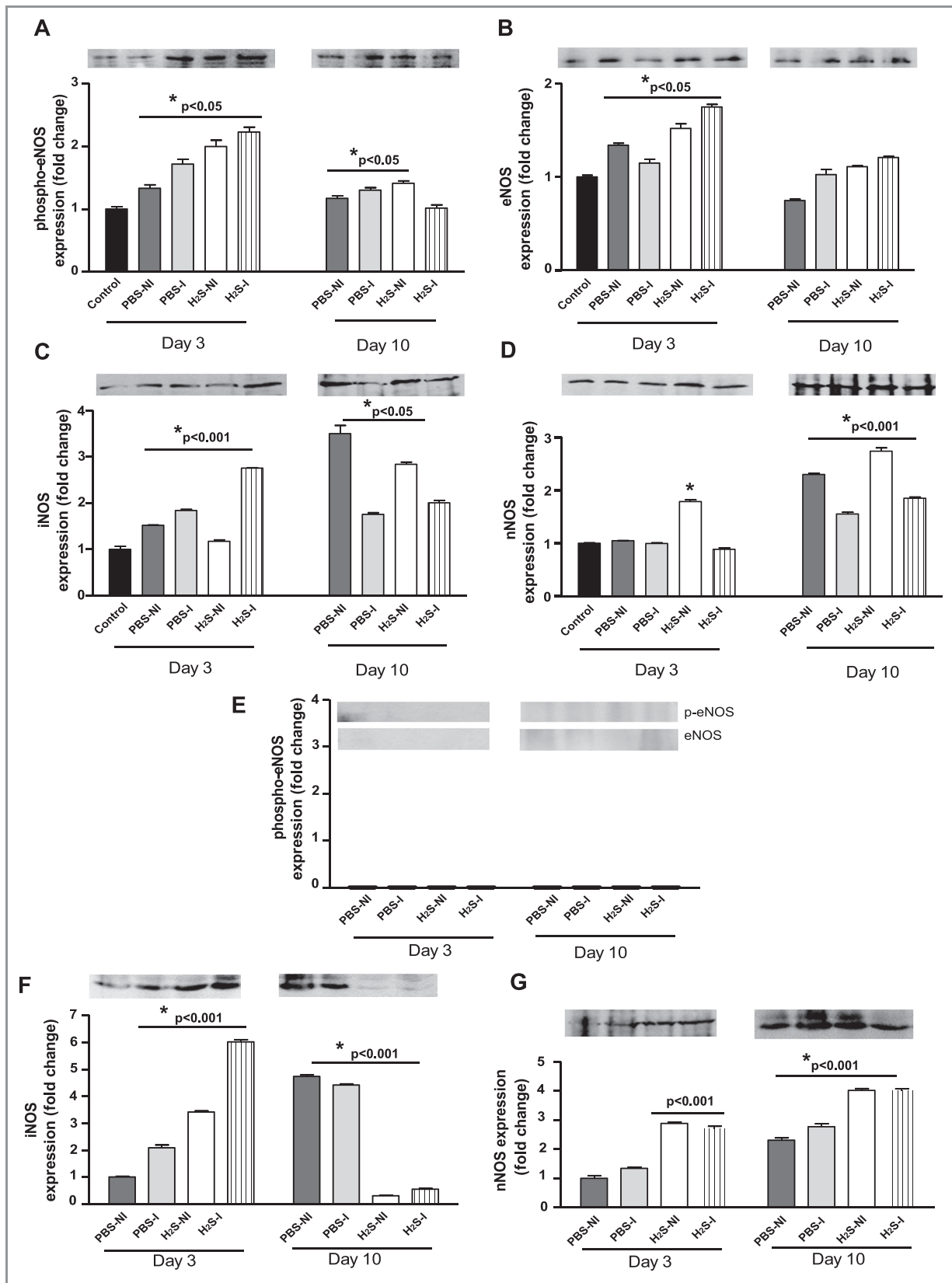


Figure 6. Western blot analyses of NOS isoforms in wild-type and eNOS^{-/-} mice. Western blots and corresponding quantification below comparing levels of phospho-eNOS (Ser 1177), total eNOS, iNOS, and nNOS, respectively, from the nonligated (NI) and ligated (I) gastrocnemius muscle tissues of wild-type mice (A to D) and eNOS^{-/-} mice (E to G), respectively. n=5 animals per genotype per time point, *P<0.05 or *P<0.001 as compared to nonligated tissues. Western blots were repeated 3 times.

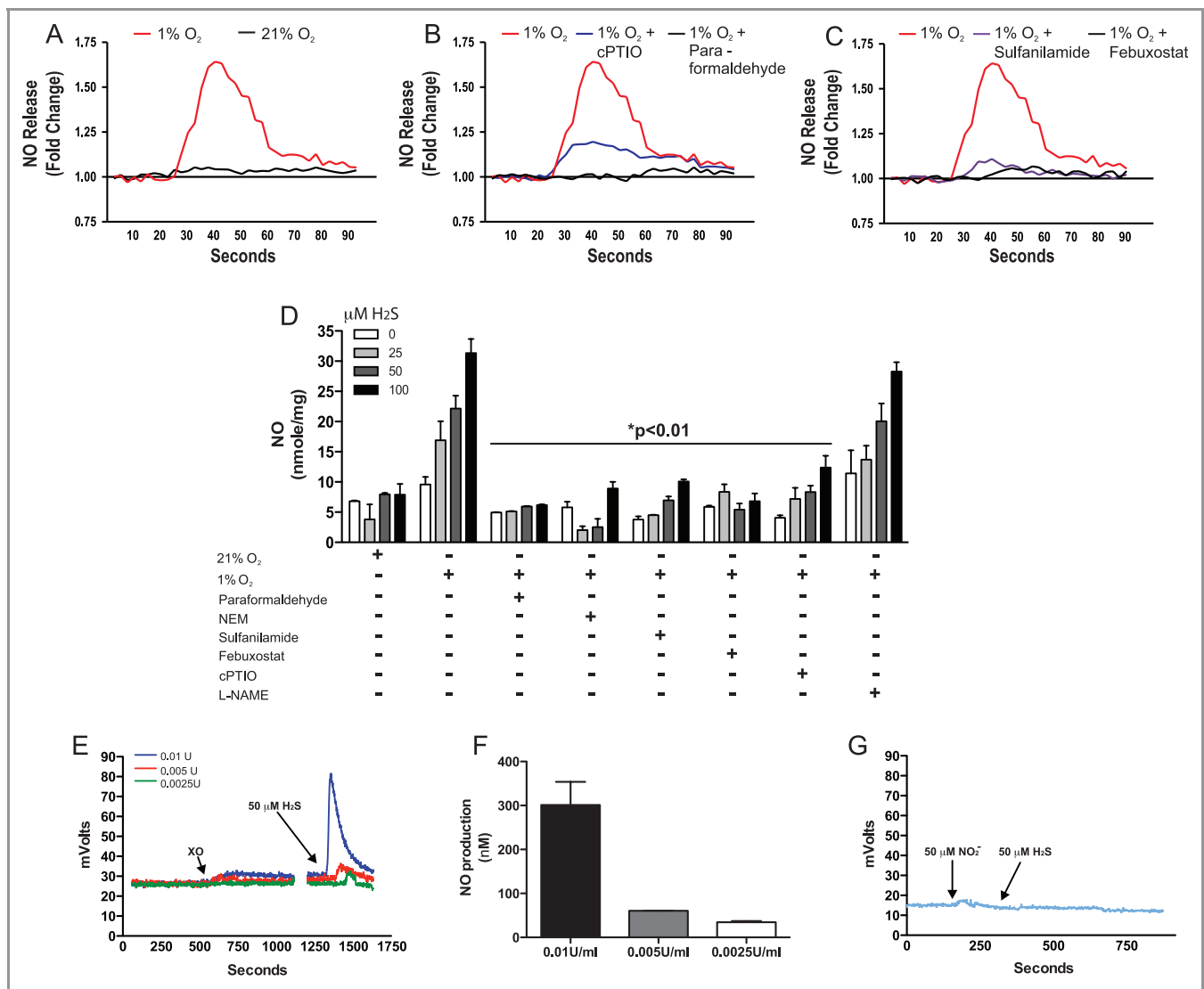


Figure 7. H₂S stimulates nitrite reduction to NO in hypoxic endothelial cells. A, Release of NO after administration of 50 μmol/L H₂S with a chemiluminescent NO analyzer under normoxic (21% O₂) and hypoxic (1% O₂) conditions. B, Release of NO after administration of 50 μmol/L H₂S after the addition of cPTIO or paraformaldehyde. C, Release of NO after administration of 50 μmol/L H₂S after treatment of cells with sulfanilamide or febuxostat. D, Amounts of released NO after administration of different doses of H₂S in the presence of paraformaldehyde (3%), NEM (10 μmol/L), sulfanilamide (1 mmol/L), febuxostat (10 nmol/L), cPTIO (200 μmol/L), or L-NAME (300 μmol/L). E through G, Interaction between H₂S and nitrite followed by amount of NO generation in the presence or absence of recombinant XO (0.0025 U, 0.005 U, and 0.01 U/mg). n=6 replicates performed in triplicate. *P<0.01 compared to PBS control data.

treated with sodium sulfide to examine NO production. Figure 7A shows the release of NO after administration of 50 μmol/L sodium sulfide to either hypoxic or normoxic endothelial cells. H₂S significantly increased NO release from hypoxic but not normoxic cultured endothelium over a 1-minute period. Studies were then performed to confirm that the H₂S-dependent chemiluminescent peak from hypoxic endothelium was indeed NO. Figure 7B shows that the NO scavenger cPTIO (200 μmol/L) significantly blunted H₂S-mediated NO formation. Moreover, protein fixation with 3% paraformaldehyde treatment of hypoxic endothelial cells also prevented H₂S-dependent NO formation, indicating that H₂S-

mediated NO production involves protein function. Figure 7C further demonstrates that H₂S-mediated NO formation is nitrite dependent, because sulfanilamide (1 mmol/L) treatment of hypoxic endothelial cells completely prevented NO formation. Treatment with febuxostat (10 nmol/L), a XO inhibitor, blocked H₂S-mediated NO formation, implicating XO-mediated nitrite reduction to NO. Figure 7D reports quantitative measurement of NO formation from dose-dependent treatment (25 to 100 μmol/L) of H₂S on hypoxic versus normoxic endothelial cells coupled with various inhibitors of specific molecular targets. Importantly, inhibition of protein thiol modification with NEM, XO with febuxostat, nitrite

availability with sulfanilamide, NO bioavailability with cPTIO, or protein fixation with paraformaldehyde all significantly blocked NO production at a range of H₂S concentrations. In contrast, L-NAME treatment did not significantly block H₂S-mediated NO formation.

To confirm that H₂S augments XO-mediated nitrite reduction to NO, we performed recombinant XO protein experiments in conjunction with NO-chemiluminescent analysis. Figure 7E shows NO-chemiluminescent traces from the addition of different concentrations of recombinant XO protein to a solution of 50 μmol/L nitrite anion, followed by administration of 50 μmol/L sodium sulfide. Resulting traces reveal a slight increase in NO production upon addition of the highest XO concentration (0.01 U) (as expected) that is followed by a significant spike in NO formation upon addition of sulfide. Figure 7F reports the amounts of NO generation upon addition of 50 μmol/L sodium sulfide to different XO concentrations in the presence of nitrite. Sulfide robustly catalyzes the reaction of 0.01 U of XO to generate ≈300 nmol/L NO from nitrite reduction. Importantly, equimolar (50 μmol/L) addition of sodium sulfide to sodium nitrite in Hank's balanced salt solution alone did not increase NO formation (Figure 7G).

Figure 8 reports the effects of H₂S on phospho-eNOS and total eNOS as well as iNOS and nNOS expression in normoxic versus hypoxic endothelial cells. PBS or 50 μmol/L sodium sulfide was added to the cells, which were incubated for 5, 15, 30, and 60 minutes. H₂S significantly increased phospho-eNOS levels by 60 minutes, which is consistent with findings from Yusof et al (Figure 8A).²² However, H₂S did not significantly alter eNOS phosphorylation under hypoxic conditions at any time point examined (Figure 8B). Moreover, H₂S stimulated moderate (≈50%) differences in iNOS expression under normoxic conditions that was not observed under hypoxia (Figure 8C and 8D). Changes in nNOS expression were decreased by H₂S stimulation under both normoxic and hypoxic conditions (Figure 8E and 8F). Finally, endothelial XO expression and activity was increased under hypoxic conditions, confirming the availability of this key enzyme for H₂S-mediated nitrite reduction to NO (Figure 8G). Together, these data clearly demonstrate that H₂S quickly augments NO formation in hypoxic endothelial cells within minutes after its addition in a XO-dependent manner and that H₂S does not significantly increase eNOS expression under hypoxic conditions.

Increased Xanthine Oxidase Activity Is Required for H₂S-Dependent Restoration of Blood Flow in Hind-Limb Ischemia

Having observed that sulfide can increase XO nitrite reductase activity under hypoxic conditions, we examined whether H₂S therapy altered XO activity in vivo and whether XO

activity was important for H₂S-mediated ischemic limb reperfusion. Figure 9A and 9B show that XO activity was significantly increased in H₂S-treated ischemic tissues of wild-type and eNOS-knockout mice. Treatment with the XO inhibitor febuxostat (5 mg/kg twice a day) blocked H₂S-mediated increases in ischemic tissue XO activity, as well as H₂S-mediated restoration of ischemic hind-limb blood flow, in both wild-type and eNOS^{-/-} mice (Figure 9C and 9D). Moreover, febuxostat also blunted H₂S-mediated increases in angiogenic activity in wild-type and eNOS^{-/-} mice (Figure 9E and 9F), demonstrating that XO is required to stimulate the H₂S-mediated ischemic angiogenesis.

H₂S Increases Ischemic Tissue Hypoxia-Inducible Factor-1α VEGF Levels in a NO-Dependent Manner

NO and hypoxia-inducible factor (HIF)-1α are known to enhance VEGF expression during tissue ischemia.^{23,24} We next examined downstream molecular mechanisms of H₂S treatment on endothelial cell HIF-1α activation in an oxygen-dependent manner. H₂S treatment of normoxic endothelial cells did not alter HIF-1α activity over a 12-hour time period (Figure 10A). However, 50 μmol/L H₂S stimulated a 5-fold increase in HIF-1α activation under hypoxic conditions (Figure 10B). HIF-1α activation plays an important role in regulating hypoxic endothelial cell proliferation responses and has been shown to be activated by NO.^{25,26} We found that H₂S stimulated a moderate increase in endothelial cell proliferation at 21% O₂ (Figure 10C) but that H₂S elicited a robust 7-fold increase in endothelial cell proliferation under hypoxia (1% O₂) that was significantly inhibited by HIF-1α small interfering RNA or cPTIO. Moreover, we found that H₂S therapy significantly increased HIF-1α expression in ischemic tissues, which was completely attenuated by XO inhibition with febuxostat (Figure 10D and 11). Together, these data demonstrate that H₂S increases HIF-1α expression and function under low-oxygen conditions that involve XO activity and NO formation.

Next, we examined whether H₂S augments ischemic tissue angiogenesis and reperfusion involving VEGF production. Figure 12A shows gastrocnemius tissue VEGF levels at day 7 in either PBS-treated or 0.5 mg/kg H₂S-treated animals. H₂S significantly augmented ischemic tissue VEGF levels over that of PBS treatment, as measured by ELISA. H₂S further increased ischemic tissue levels of VEGF over that of PBS treatment at day 10 (Figure 12B). Importantly, combined treatment of H₂S plus cPTIO significantly blocked H₂S induction of VEGF during tissue ischemia, indicating that NO is crucially important for H₂S augmentation of VEGF during ischemia (Figure 12C). We also discovered that H₂S could increase ischemic tissue VEGF expression in

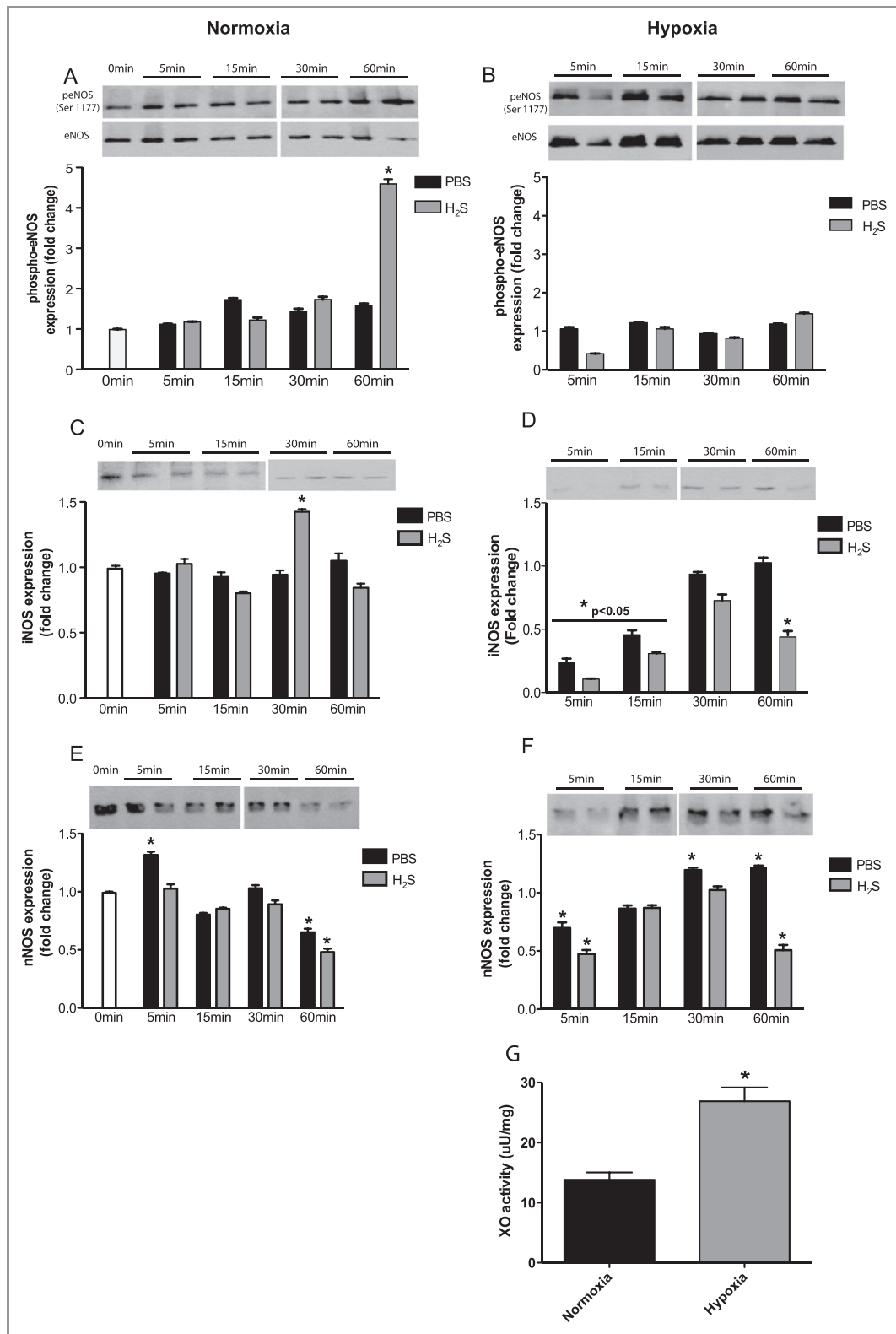


Figure 8. H₂S effects on eNOS expression and activation in vitro. Mouse endothelial cells were cultured under normoxic or hypoxic conditions and then treated with 50 μ mol/L H₂S and used for NOS Western blot analysis at different time points. A and B, Phospho/total eNOS under normoxia and hypoxia with PBS or sulfide stimulation, respectively. C and D, iNOS expression under normoxia and hypoxia with PBS or sulfide stimulation, respectively. E and F, nNOS expression under normoxia and hypoxia with PBS or sulfide stimulation, respectively. G, Endothelial XO activity under normoxia and hypoxia. n=5, Western blots were repeated in triplicate, **P*<0.05 0 min PBS or Normoxia versus H₂S per time point. Solid line with significance indication illustrates differences between respective time points.

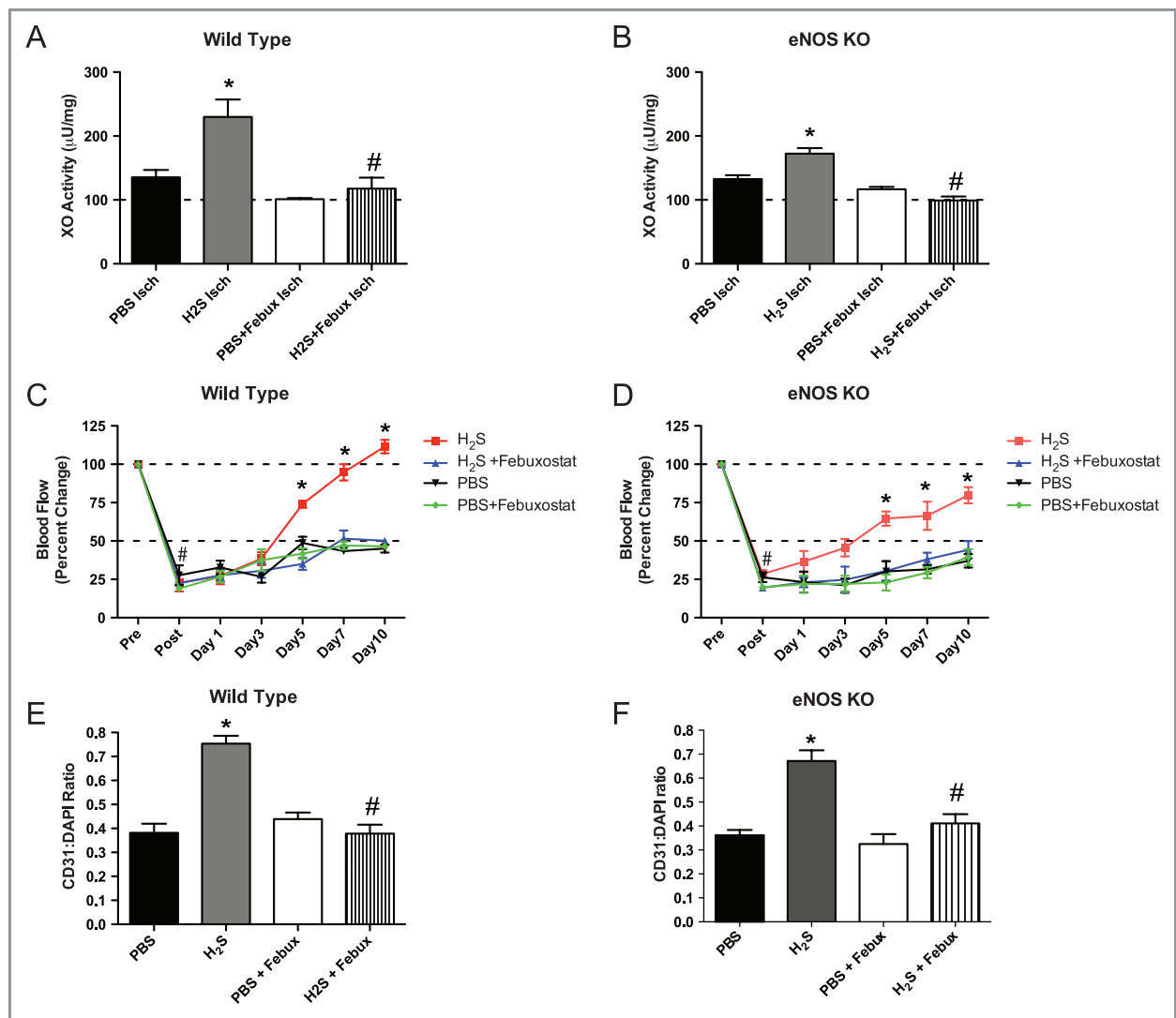


Figure 9. H₂S increases ischemic tissue XO activity that regulates ischemic tissue reperfusion and vascular remodeling. A and B, XO activity after H₂S therapy in wild-type and eNOS^{-/-} mice 10 days after ligation. C and D, Blood flow recovery after febuxostat in wild-type and eNOS^{-/-} mice in different time points. E and F, Angiogenesis index in wild-type and eNOS^{-/-} mice 10 days after ligation. n=5 animals, per cohort, **P*<0.05 compared to PBS ischemic tissue data and #*P*<0.05 compared to H₂S ischemic tissue data, or before and after ligation.

eNOS-deficient mice, which was blocked by cPTIO but not L-NAME (Figure 12D). These data reinforce the importance of H₂S-dependent nitrite reduction to NO as a key regulator of ischemic tissue VEGF induction. Lastly, we found that scavenging VEGF with the VEGF₁₆₄ aptamer (Macugen) (50 mg/kg) could significantly prevent H₂S-mediated ischemic limb reperfusion in wild-type animals (Figure 12E). VEGF₁₆₄ aptamer also significantly blunted H₂S-mediated VEGF bioavailability in ischemic tissues (Figure 12F). Heat-denatured VEGF₁₆₄ aptamer (D-VEGF aptamer) control treatments were unable to block H₂S-mediated ischemic limb reperfusion or VEGF expression, revealing that H₂S selectively stimulates VEGF expression in ischemic tissues in a NO-dependent manner.

H₂S Increases Diabetic Ischemic Hind-Limb Reperfusion and Angiogenesis in a VEGF-Dependent Manner

Impaired ischemic vascular remodeling during diabetes in Db/Db mice involves defective VEGF expression and signaling,²⁷ with aged diabetic Db/Db mice manifesting severe defects in ischemic angiogenic responses.^{28,29} We next examined whether exogenous H₂S therapy could augment ischemic limb reperfusion and vascular growth in 9-month-old, aged Db/Db mice subjected to femoral artery ligation. To emulate therapeutic situations, we delayed H₂S administration until day 5 after ligation, as we have recently reported, thus allowing the establishment of tissue ischemia.³⁰ Figure 13A

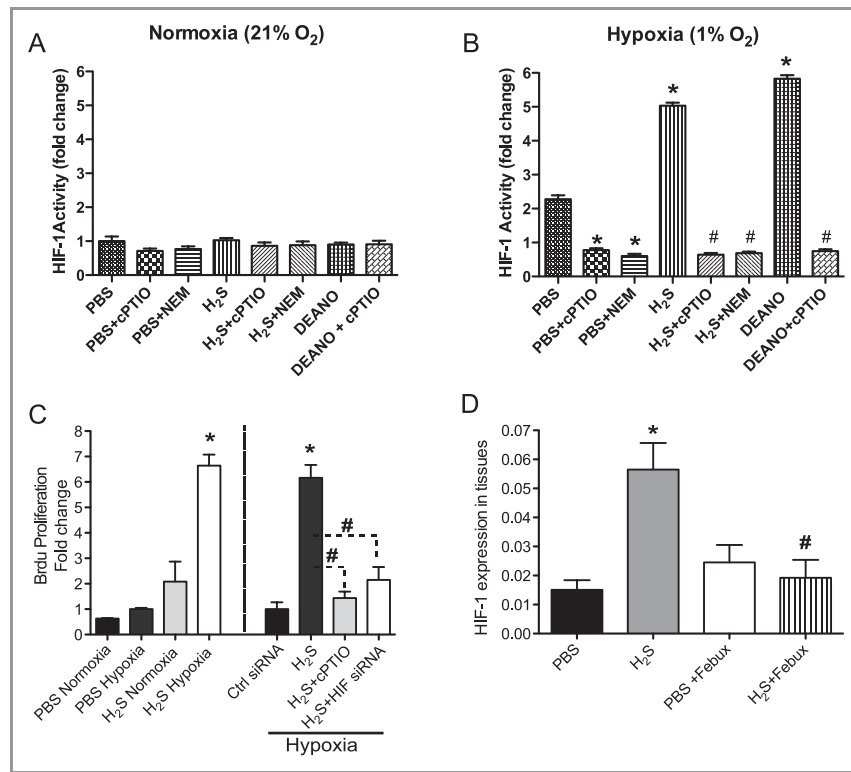


Figure 10. H₂S stimulates hypoxic endothelial cell proliferation in a NO–HIF-1 α –dependent manner. A, Endothelial bromodeoxyuridine (BrdU) incorporation after H₂S dose–dependent treatment in normoxic (21% O₂) conditions. B, Endothelial BrdU incorporation after H₂S dose–dependent treatment in hypoxic (1% O₂) conditions. C, Effect of 50 μ mol/L sodium sulfide on HIF-1 α activity under normoxic (21% O₂) conditions. D, Effect of sodium sulfide on HIF-1 α activity under hypoxic (1% O₂) conditions in vitro. E, Effect of NEM, cPTIO, or HIF-1 α small interfering RNA (siRNA) knockdown on H₂S-mediated hypoxic endothelial cell proliferation. n=6 replicates performed in triplicate, *P<0.05 compared to PBS, #P<0.05 compared to H₂S or diethylamine NONOate (DEANO) treatment alone.

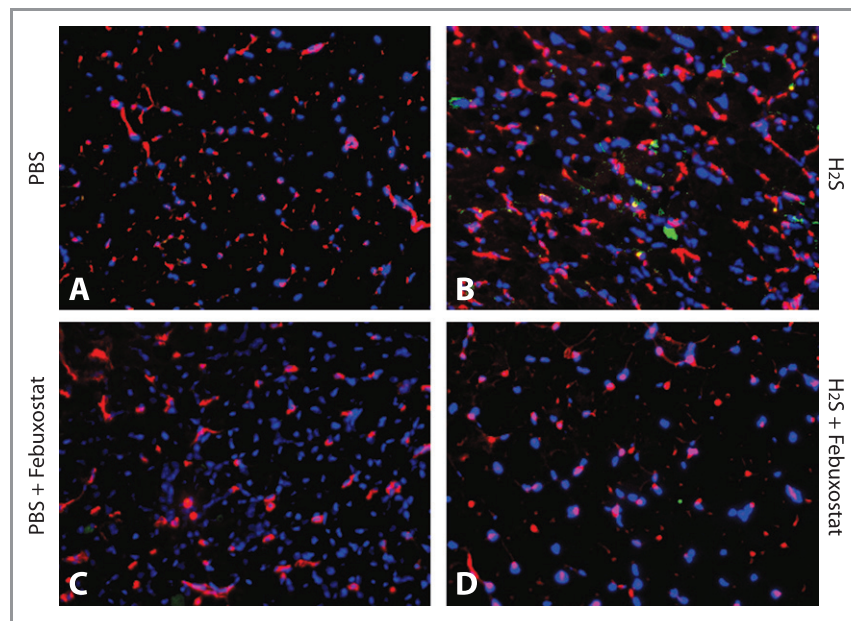


Figure 11. H₂S increases HIF-1 α expression in ischemic tissues in an XO-dependent manner. Tissues were stained with anti-CD31 (red), anti-HIF-1 α (green), and DAPI nuclear counterstain (blue). A, CD31 and HIF-1 α staining in PBS-treated ischemic tissue at day 10. B, CD31 and HIF-1 α staining in H₂S-treated ischemic tissue at day 10. C, CD31 and HIF-1 α staining in PBS+febuxostat treatment. D, CD31 α and HIF-1 α staining in H₂S+febuxostat treatments.

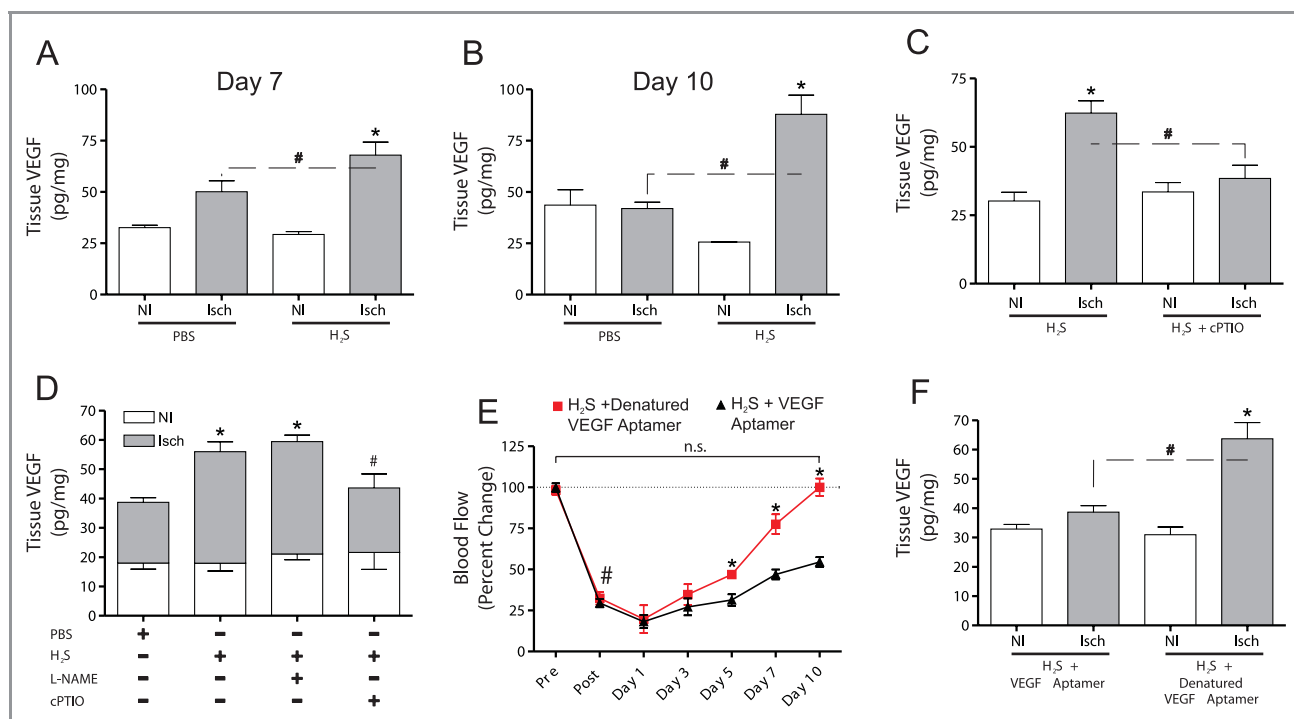


Figure 12. H₂S increases ischemic tissue VEGF expression that regulates reperfusion. A, Tissue concentrations of VEGF in ischemic and nonischemic tissues of 0.5 mg/kg sodium sulfide–treated animals at 7 days after ligation. B, Tissue concentrations of VEGF in ischemic and nonischemic tissues of sodium sulfide–treated animals at 10 days after ligation. C, Effect of H₂S+cPTIO on VEGF expression in ischemic and nonischemic tissues. D, Concentrations of VEGF protein in tissues from H₂S-treated eNOS^{-/-} mice in the presence and absence of L-NAME or cPTIO. E, Ischemic limb blood flow values of wild-type animals treated with H₂S+VEGF₁₆₄ aptamer or denatured VEGF₁₆₄ aptamer. F, Tissue VEGF levels of wild-type animals treated with H₂S+VEGF₁₆₄ aptamer or denatured VEGF₁₆₄ aptamer. n=12 animals per experimental cohort, **P*<0.05 compared to nonischemic, or VEGF₁₆₄ aptamer data; #*P*<0.05 ischemic limb comparison, or before and after ligation.

and 13B illustrate that delayed H₂S therapy significantly restored ischemic hind-limb perfusion and capillary-to-myofiber ratios compared to PBS control treatment. Figure 13C and 13D illustrate that cotreatment with H₂S plus a VEGF₁₆₄-inhibiting aptamer significantly prevented H₂S-mediated ischemic limb reperfusion and increased ischemic tissue VEGF protein levels compared to denatured VEGF₁₆₄ control aptamer treatment. Finally, Figure 13E and 13F clearly demonstrate that H₂S plus VEGF₁₆₄ aptamer treatment significantly prevented increased ischemic vascular density and proliferation. Together, these data highlight the potent therapeutic effect of H₂S on diabetic ischemic vascular remodeling and tissue reperfusion, which depends on increased VEGF expression and angiogenic activity.

Discussion

H₂S–NO pathway interactions have been suggested, although specific mechanistic information on how these gasotransmitters influence one another remains poorly understood. Our findings provide significant insight into how H₂S affects NO metabolism responses during ischemic vascular remodeling such that (1) H₂S therapy restores ischemic hind-limb blood

flow in a NO-dependent manner, (2) H₂S increases NOS expression concomitant with stimulating XO-mediated nitrite reduction to NO in ischemic tissues, and (3) H₂S stimulates the ischemic vascular growth by augmenting the expression and activity of HIF-1 α and VEGF in a NO-dependent manner. We also clearly observed that H₂S selectively accumulates in and significantly increases NO bioavailability of chronically ischemic tissue. These beneficial effects of H₂S on ischemic tissue NO levels do not critically depend on NOS activity but do significantly depend on nitrite anion reduction back to NO. These findings further define biochemical mechanisms in which H₂S increases tissue NO bioavailability that occurs during cardiovascular disease and tissue ischemia.

Exogenous H₂S has been reported to interfere with NO donor vasodilation responses, presumably through direct interactions generating unknown species.¹⁶ However, the biological effect of H₂S seems to be oxygen dependent, as it mediates aortic ring vasoconstriction at high oxygen tensions but vasodilation at low oxygen tension.³¹ Here, we found that H₂S treatment increased ischemic vascular remodeling, consistent with previous studies; however, our results identified H₂S-dependent release of NO through nitrite reduction as a key cytoprotective mechanism during tissue ischemia.³²

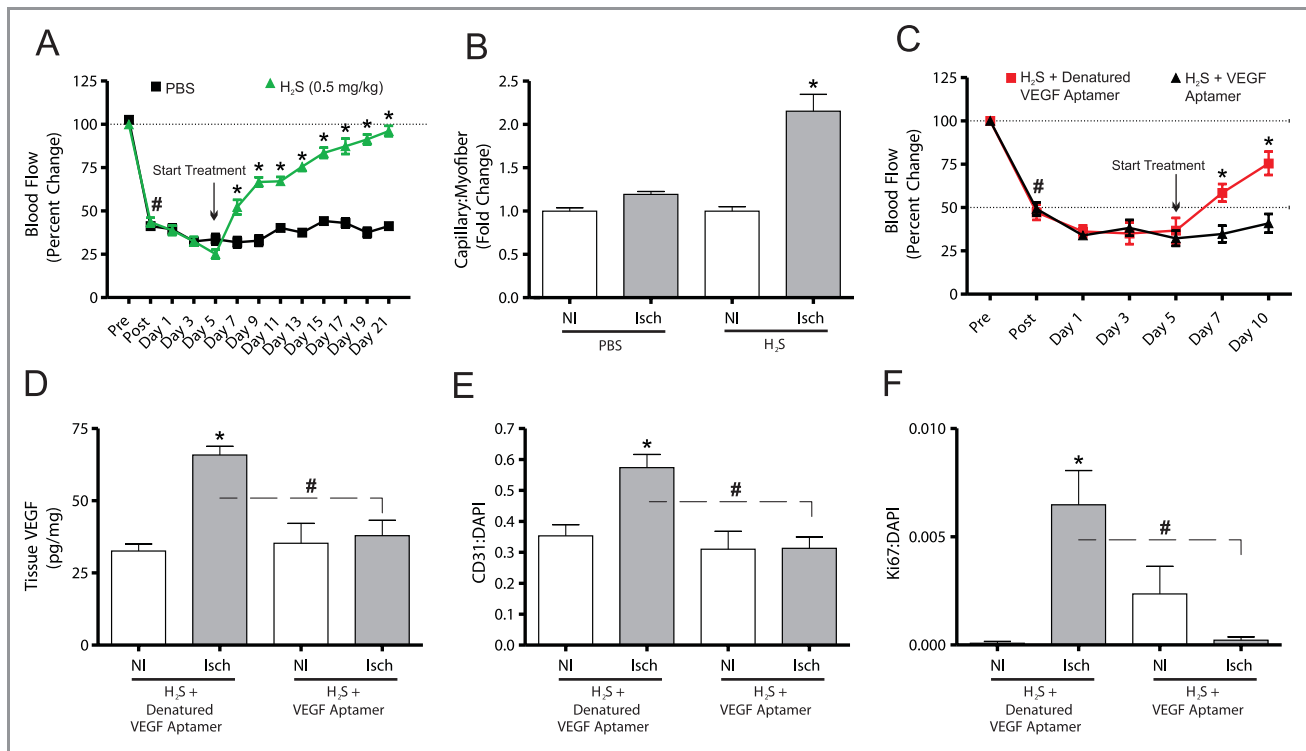


Figure 13. H₂S restores established diabetic ischemic limb reperfusion and angiogenic activity in a VEGF-dependent manner. A, Ischemic hind-limb blood flow changes in 9-month-old diabetic mice subjected to femoral artery ligation followed by delayed 0.5 mg/kg sodium sulfide or PBS control therapy. B, Capillary-to-myofiber ratio change with sodium sulfide versus PBS therapy between nonischemic and ischemic gastrocnemius muscle tissue. C, Ischemic hind-limb blood flow in diabetic animals treated with H₂S+VEGF₁₆₄ aptamer (25 mg/kg IM injection, twice daily) or heat-denatured VEGF₁₆₄ aptamer. D, Tissue VEGF levels in H₂S-treated diabetic animals with VEGF₁₆₄ aptamer or heat-denatured VEGF₁₆₄ aptamer. E, Graphical representation of the CD31:DAPI ratio demonstrating vascular density in different gastrocnemius tissues from mice treated with H₂S + denatured VEGF aptamer and H₂S + VEGF aptamer. F, Graphical representation of Ki67:DAPI ratio demonstrating proliferation in different gastrocnemius tissues from mice treated with H₂S + denatured VEGF aptamer and H₂S + VEGF aptamer. For A, B, and C: n=8 animals per experimental cohort, * $P \leq 0.05$ compared to PBS control or VEGF₁₆₄ aptamer data; # $P \leq 0.05$ ischemic limb comparison, or before and after ligation. For D, E, and F: n=8 animals per experimental cohort, * $P \leq 0.05$ compared to nonischemic limb H₂S + denatured VEGF₁₆₄ aptamer data; # $P \leq 0.05$ ischemic limb comparison.

Previous studies from Cai et al and Papapetropoulos et al did not observe H₂S-mediated NO formation in their angiogenesis studies.^{32,33} Conversely, Coletta and coworkers recently have reported that mutual interaction between H₂S and NO might be important for physiological control of vascular function.³⁴ Specifically, they reported that H₂S modulates wound healing responses, vasodilation, and vessel sprouting, involving activation of PI3K/Akt/phospho-eNOS and NO bioavailability. However, no studies have examined molecular mechanisms of H₂S on NO metabolism under chronic ischemia/hypoxic conditions. Previously, Wang et al reported that H₂S therapy during tissue ischemia did not alter plasma NO levels³⁵; however, this study only examined plasma nitrite/nitrate levels with the Griess reaction methodology, which is unable to detect physiological changes in plasma or tissue NO metabolites (eg, NO, nitrosothiols, nitrosoheme). Using the NO-chemiluminescent measurement technique, we clearly observed multiple effects of exogenous

H₂S on NO formation involving different mechanisms and metabolite species. It has been suggested that some of the beneficial cardiovascular effects of H₂S could involve eNOS activation and expression.^{15,17} However, our data indicate that eNOS expression or function is not essential for H₂S-mediated ischemic vascular remodeling or NO formation, revealing that H₂S stimulation of XO-dependent nitrite reduction to NO acts as an alternative mechanism to maintain NO bioavailability. Our findings are consistent with classical studies from the Massey laboratory that demonstrated the importance of hydropersulfide formation at the active site of XO in regulating its activity, such that sodium sulfide was shown to increase XO protein activity, which we confirmed in vivo and in vitro.^{36,37}

We and others have reported that nitrite anion reduction to NO mediates cytoprotection during acute ischemia-reperfusion injury and chronic tissue ischemia.^{19,38} We previously have reported that nitrite-dependent NO formation rapidly

augments ischemic hind-limb reperfusion and ischemic vascular remodeling.¹⁹ The biological potency of H₂S treatment for chronic tissue ischemia is similar to that of sodium nitrite, which suggests that a major pathway of the beneficial effects of H₂S might occur via nitrite-dependent NO formation apart from other previously defined effects, such as antioxidant, signaling (K-ATP or phosphodiesterase 5), and transcription factor changes. Figure 14 supports this hypothesis, because blockade of K-ATP channels (known to be activated by H₂S) with glibenclamide does not prevent H₂S-increased ischemic hind-limb blood flow or ischemic vascular remodeling. Taken together, it appears that XO-mediated nitrite reduction to NO plays a key role in mediating the beneficial effects of H₂S for ischemic vascular remodeling.

NO formation through XO-mediated nitrite reduction critically serves to maintain NO bioavailability in addition to NOS-dependent mechanisms.³⁹ Thus, XO is an important source for NOS-independent NO generation under conditions in which NOS activity could be limiting, and our findings clearly show that sulfide can stimulate recombinant XO-dependent nitrite reduction to NO. This most likely occurs through posttranslational modification of XO (presumably sulfhydrylation) that is currently being studied in greater detail. Nonetheless, *in vitro* and *in vivo* XO-inhibition studies revealed XO activation to be a dominant mediator of ischemic tissue

NO formation because febuxostat, but not L-NAME inhibition of NOS activity, completely prevented the beneficial effects of H₂S in eNOS^{-/-} mice. This is confirmed by the observation that XO-mediated H₂S-induced NO production can enhance cGMP production in ischemic tissue of eNOS^{-/-} and wild-type mice. Future studies will further examine how H₂S affects XO activity *in vivo* and *in vitro* regulating NO production and bioavailability.

Recent reports suggest that H₂S could positively regulate HIF-1 α activity.^{40,41} These studies showed that H₂S-enhanced hypoxia induced HIF-1 α and VEGF expression in vascular smooth muscle cells, which enhanced endothelial cell viability. Our findings further advance our understanding of H₂S and HIF-1 α because H₂S stimulation of endothelial cell proliferation was maximal under hypoxia that critically required NO-dependent HIF-1 α activity. Endogenous NO is known to stimulate VEGF synthesis and VEGF-induced angiogenesis that is blunted by NOS inhibition.⁴²⁻⁴⁴ Likewise, angiogenesis is impaired when NO bioavailability is attenuated, as seen during hind-limb ischemia in eNOS-knockout mice that is not significantly reversed by administration of VEGF.⁴⁵ Moreover, NO along with cGMP production exerts these effects by increasing expression of VEGF.^{42,46,47} Our results are in agreement with these previous reports and reveal that H₂S increases VEGF expression in eNOS^{-/-} ischemic tissues in a NO-dependent manner that can stimulate angiogenesis in ischemic tissues. The implications of our results are striking, as ischemic VEGF induction in eNOS^{-/-} mice is ultimately NO dependent, which reinforces the importance of crosstalk between H₂S and NO metabolism pathways.

In conclusion, we have discovered that H₂S selectively augments NO production and associated downstream vascular growth and remodeling in chronically ischemic tissues by influencing NOS expression and critically stimulating nitrite reduction to NO via XO. H₂S has been proposed to be a novel therapeutic for ischemia-reperfusion injury; however, our data also demonstrate its benefit for NO-dependent ischemic vascular disorders that have yet to achieve broad clinical resolution.⁴⁸ Further studies are needed to better understand the biological and therapeutic implications of H₂S-dependent nitrite reduction to NO, including downstream signaling pathways that are activated to confer ischemic tissue cytoprotection.

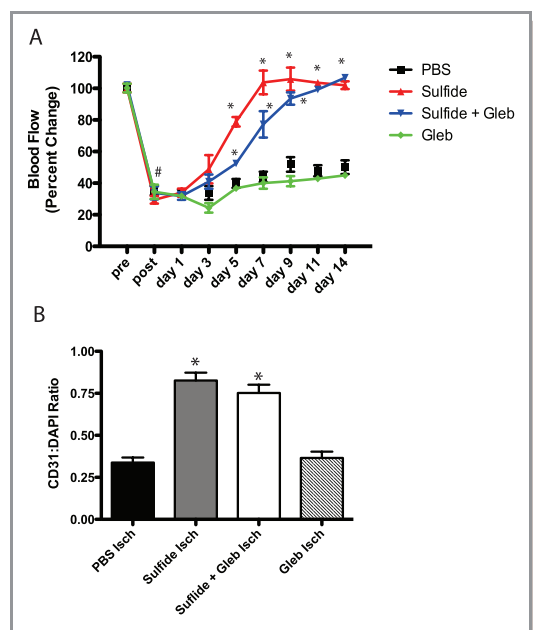


Figure 14. Inhibition of K-ATP channels on H₂S-mediated ischemic vascular remodeling. A, Effect of glibenclamide (1.4 mg/kg)-mediated K-ATP channel inhibition on H₂S-dependent ischemic limb reperfusion. B, Effect of glibenclamide plus H₂S on ischemic vascular CD31:DAPI density. n=8 animals per treatment cohort, *P<0.05 versus PBS ischemia.

Sources of Funding

This work was sponsored by NIH grants HL80482, HL113303, and DK043785 Project 4 to Dr Kevil and HL094021 to Dr Pattillo. Dr Bir is funded by a fellowship from the Malcolm Feist Cardiovascular Research Endowment, LSU Health Sciences Center–Shreveport.

Disclosures

C.G.K is a participant on a pending US patent (No. 61/003150) for the use of nitrite salts in chronic tissue ischemia and has a commercial interest in Theravasc, Inc. The other authors have nothing to disclose.

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