Hydrogen Sulfide Contributes to Retinal Neovascularization in Ischemia-Induced Retinopathy

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PURPOSE. Hydrogen sulfide (H2S) is an endogenous gaseous signaling molecule with significant pathophysiological importance, but its role in retinal neovascular diseases is unknown. Hydrogen sulfide is generated from L-cysteine by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and/or 3-mercaptoppyruvate sulfurtransferase (3-MST). The aim of this study was to investigate the role of H2S in retinal neovascularization (NV) in ischemia-induced retinopathy.

METHODS. Studies were performed in a murine model of oxygen-induced retinopathy (OIR). Hydrogen sulfide was detected with a fluorescent assay. Western blots and immunohistochemistry were used to assess the changes of H2S-producing enzymes. Gene deletion and pharmacologic inhibition were used to investigate the role of H2S in retinal NV.

RESULTS. Hydrogen sulfide production was markedly increased in retinas from OIR mice compared with those from room air (RA) controls. Cystathionine-β-synthase and CSE were significantly increased in OIR retinas, whereas 3-MST was not changed. Cystathionine-β-synthase was expressed throughout the neuronal retina and upregulated in neurons and glia during OIR. Cystathionine-γ-lyase was also localized to multiple retinal layers. Its immunoreactivity was prominently increased in neovascular tufts in OIR. Pharmacologic inhibition of CBS/CSE or genetic deletion of CSE significantly reduced retinal NV in OIR.

CONCLUSIONS. Our data indicate that the H2S-generating enzymes/H2S contributes to retinal NV in ischemia-induced retinopathy and suggest that blocking this pathway may provide novel therapeutic approaches for the treatment of proliferative retinopathy.

Keywords: hydrogen sulfide, neovascularization, oxygen-induced retinopathy

Ischemia-induced pathologic retinal neovascularization (NV) is a common cause of irreversible vision loss, occurring in conditions such as proliferative diabetic retinopathy and retinopathy of prematurity.1–3 Treatment for pathologic retinal NV includes panretinal laser photocoagulation, which destroys neurons to lower the total retinal metabolic demand and alleviate ischemia.5 Intravitreal injections of anti-vascular endothelial growth factor (VEGF) are also used to treat pathologic NV but their therapeutic effect is short-lived.4 In order to develop novel therapeutic strategies for ischemia-induced retinopathy, it is necessary to elucidate the mechanisms and identify novel mediators involved in the progression of retinal pathologic NV.

Hydrogen sulfide (H2S) is a crucial gaseous signaling molecule that is involved in many physiological and pathologic conditions.5–21 Three H2S-generating enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptoppyruvate sulfurtransferase (3-MST), are responsible for H2S production in various types of tissues.22,23 The role of H2S in retinal pathophysiological changes has been appreciated in the past decade. Studies discovered several protective properties of H2S in a variety of conditions, including protecting retina from light-induced degeneration by suppression of Ca2+ influx,9 attenuating excitotoxic neurotransmission,9 protecting retinal neuronal cell apoptosis against NMDA or retinal ischemia-reperfusion–induced injury via its antioxidative activity,11,12,24 inducing retinal vascular relaxation,25,26 and alleviating retinal oxidative stress and inflammation in a rat model of streptozotocin-induced diabetes.27 In spite of this progress, the function of H2S in neovascular disease in the retina is unknown, although increased plasma and vitreous H2S has been found in patients with proliferative diabetic retinopathy,28 and H2S exhibits proangiogenic property in organs where ischemic vessel growth is beneficial.15–20 In this study, we determined the role of H2S-generating enzymes/H2S pathway in retinal NV using a mouse model of oxygen-induced retinopathy.

METHODS

Treatment of Animals

All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by The University of Texas Medical Branch Institutional Animal Care and Use
Committee. CSE+/− mice were generated as described previ-
ously.13,17 CSE−/− and CSE+/− littersmates were obtained by
crossing CSE+/− and CSE−/− mice, and CSE−/− and CSE+/−
littersmates were obtained by crossing CSE−/− and CSE+/− mice.
Oxygen-induced retinopathy (OIR) was induced by exposing
litters to 70% oxygen from postnatal day (P)7 to P12, followed
by a return to room air from P12 to P17. Age-matched C57BL-6
mice were kept in room air. From P12 to P17, the animals were
anesthetized and their retinas were collected for morphologic
or molecular studies. In a subset of wild-type mice exposed to
OIR, a frequently used CBS and CSE inhibitor, aminooxyacetic
acid (AOAA)29 (Sigma-Aldrich Corp., St. Louis, MO, USA), or
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**Retinal H2S Detection**

Retinal H2S was detected using previously described fluores-
cent methods with minor modifications.30 Briefly, isolated retinas
were homogenized in a non-denaturing lysis buffer (50
mM Tris-HCl pH = 8.0, 150 mM NaCl, 1% Nonidet P-40, 1%
Triton X-100, protease inhibitors) on ice for 20 minutes,
followed by centrifugation at 14,000g for 15 minutes at 4°C.
The protein concentration was determined by bicinchoninic
acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL,
USA). The reaction mixture contained the protein cell extract,
50 mM Tris-HCl (Sigma-Aldrich Corp.), 1 mM L-cysteine (Sigma-
Aldrich Corp.), 1 mM homocysteine (Sigma-Aldrich Corp.), 50
μM pyridoxal-5’-phosphate (MP Biomedicals, Santa Ana, CA,
USA), and 10 μM H2S probe 7-azido-4-methylcoumarin (7-Az)
(Santa Cruz Biotechnology, Santa Cruz, CA, USA). Reactions
were incubated for 2 hours at 37°C and fluorescence was
measured with a Synergy H1 Hybrid Reader (Bio-tek Instru-
ments, Winooski, VT, USA) using ex = 365 nm, em = 450 nm. A
linear standard curve was generated by adding freshly prepared
sodium hydrosulfide (NaH2S) to the fluorescent assay medium.

**Plasma H2S Detection**

Animals were anesthetized with isoflurane, and a syringe with
26-gauge needle was inserted into the heart to draw blood for
H2S analysis. Plasma (20 μL) was collected after centrifuging
and incubated with 7-Az for 15 minutes at 37°C, and then
fluorescence was measured with a Synergy H1 Hybrid Reader.
Fluorescent product was calculated by subtracting background
fluorescence of plasma. Hydrogen sulfide detection was
performed in duplicate for each blood sample.

**Western Blotting**

Isolated retinas were homogenized in radioimmunoprecipitation
assay (RIPA) buffer (Millipore, Billerica, MA, USA) supplemented
with protease inhibitors (Roche Applied Science, Indianapolis,
IN, USA). The lysates were centrifuged to clear debris, and
protein concentration was determined by a BCA assay (Thermo
Fisher Scientific, Inc., Waltham, MA, USA). Proteins were then
separated by SDS-PAGE and blotted onto a polyvinylidene
difluoride (PVDF) membrane. The membrane was blocked and
incubated overnight at 4°C with primary antibodies against
tubulin (1:10,000; Sigma-Aldrich Corp.), CSE (1:1000; Pepro-
Tech, Rocky Hill, NJ, USA), CBS (1:1000; Abnova; Walnut, CA,
USA), or 3-MST (1:1000; Atlas Antibodies, Stockholm, Sweden).
After washing, membranes were incubated with horseshadish
peroxidase–conjugated secondary antibody (1:3000; Cell Signal-
ing, Danvers, MA, USA). Immunoreactive proteins were detected
using the enhanced chemiluminescence system (Thermo Fisher
Scientific). Protein levels were quantified via densitometry with
ImageJ software (http://imagej.nih.gov/ij/) provided in the public
domain by the National Institutes of Health, Bethesda, MD, USA).

**RNA Isolation and Quantitative Reverse Transcriptase–Polymerase Chain Reaction (qPCR)**

Total RNA was extracted from mouse retinas using RNAqueous-
4PCR kit (Invitrogen, Carlsbad, CA, USA) according to the
manufacturer’s instructions. Quantitative PCR was performed as
described before.31 Primer sequences for mouse transcripts were
as follows: angiopoietin-2 For-5’-ACA CCG AGA AGA TGG CAG
TGT-3’; angiopoietin-2 Rev-5’-CTC CGG AAC CCC TCT TTG TA-
3’; erythropoietin (EPO) For-5’-CCC CCA CGC CTC ATC TG-3’;
EPO Rev-5’-TGC CTC CCT GGC CTC TAA GA-3’; VEGF For-5’-
TAC CTC CAT GGC AAG TG-3’; VEGF Rev-5’-TCA TGG GAC
TTC TGC TCT CCT T-3’; hypoxanthine-guanine phosphoribosyl-
transferase (Hprt) For-5’-GAA AGA CTT GCT CGA GAT GTC ATG-
3’; Hprt Rev-5’-CAC ACA GAG GGC CAC AAT GT-3’. The fold
difference in various transcripts was calculated by the ΔΔCT
method using Hprt as the internal control.

**Immunostaining of Retinal Sections**

Eyeballs were fixed in 4% paraformaldehyde for 1 hour on ice,
equilibrated in 30% sucrose overnight, and embedded in optical
cutting solution temperature compound (Tissue Tek; Sakura
Finetek, Torrance, CA, USA). Retinas were cut into 10-μm
sections, permeabilized with PBS containing Triton X-100 for 15
minutes at room temperature, and blocked with blocking buffer
(BioGenex, San Ramon, CA, USA) for 1 hour. Sections were
incubated with Alexa Fluor 594–labeled goat anti-rabbit second-
ary antibody (1:1000; Invitrogen) and anti-CSE (1:400) or anti-CBS
(1:500) overnight at 4°C. After washing with PBS, sections were
incubated with Alexa Fluor 488–labeled goat anti-rabbit second-
ary antibody (1:1000; Invitrogen) at room temperature for 1
hour. To costain CBS and glial fibrillary acidic protein (GFAP),
CBS antibody–labeled sections (red) were incubated with GFAP
antibody (1:100; Dako, Carpinteria, CA, USA) conjugated with
Alexa Fluor 488 using Zenon Rabbit IgG labeling Kit (Invitrogen)
at room temperature for 2 hours. Slides were mounted with
mounting medium containing 4’,6-diamidino-2-phenylindole
(DAPI; Sigma-Aldrich Corp.) and examined with a fluorescence
microscope (Olympus, Center Valley, PA, USA). The mean
fluorescence intensity of CBS staining within the outer nuclear
layer (ONL) was measured using ImageJ software and expressed
as normalized intensity by subtracting background fluores-
cence.32 At least four fields were analyzed for each sample.

**Immunostaining on Whole-Mount Retinas**

The immunostaining in retinas was performed as previously
described.31 Briefly, after fixation in 4% paraformaldehyde,
retinas were carefully dissected from the choroid and sclera,
blocked, and permeabilized in PBS containing 5% goat serum and
1% Triton X-100 for 30 minutes. The retinas were incubated with
Alexa 594–labeled isoelectin B4 (Griffonia simplicifolia; 1:200; Invitrogen) and anti-CSE (1:400) or anti-CBS
(1:500) overnight at 4°C. After washing with PBS, mounted on
microscope slides in mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA, USA), and
examined with a fluorescence microscope. Vascular obliteration
area and neovascular tuft area were quantified in a semi-
automated manner that has been described in detail.33

**Statistical Analysis**

The results are expressed as mean ± SEM. Group differences
were evaluated by using post hoc Student’s t-test, and results
were considered significant at P < 0.05.
findings suggest that the increased production of H\textsubscript{2}S in OIR is mediated by CBS and CSE. 

To determine if H\textsubscript{2}S is implicated in OIR, we examined H\textsubscript{2}S production in retinas from OIR mice and room air (RA) control mice. Retinal lysates were incubated with fluorogenic probe 7-Az, which rapidly reacts with H\textsubscript{2}S and is reduced to produce the fluorescent 7-amino-4-methylcoumarin (AMC). The intensity of fluorescence linearly correlates with the amount of H\textsubscript{2}S. This method is now widely used to detect H\textsubscript{2}S production.

Hydrogen sulfide can be generated by CBS, CSE, and 3-MST. To investigate the mechanisms by which H\textsubscript{2}S production was upregulated in OIR, we measured the levels of CBS, CSE, and 3-MST by Western blot. We found that all three enzymes were expressed in the retina (Fig. 2). Compared with RA controls, CBS was significantly upregulated by 3-fold in retinas from OIR mice and room air (RA) control mice. Retinal lysates were incubated with fluorogenic probe 7-Az, which rapidly reacts with H\textsubscript{2}S and is reduced to produce the fluorescent 7-amino-4-methylcoumarin (AMC). The intensity of fluorescence linearly correlates with the amount of H\textsubscript{2}S. This method is now widely used to detect H\textsubscript{2}S production.

To investigate the mechanisms by which H\textsubscript{2}S production was upregulated in OIR, we measured the levels of CBS, CSE, and 3-MST by Western blot. We found that all three enzymes were expressed in the retina (Fig. 2). Compared with RA controls, CBS was significantly upregulated by approximately 2.5-fold and CSE was increased slightly in OIR retinas, whereas the level of 3-MST was not altered. These findings suggest that the increased production of H\textsubscript{2}S in OIR is mainly mediated by CBS and CSE.

**Expression of H\textsubscript{2}S-Producing Enzymes Is Altered in Retinas From Oxygen-Induced Retinopathy**

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**CBS and CSE Are Localized in Different Retinal Cells in Oxygen-Induced Retinopathy**

We next determined the retinal localization of CBS and CSE. In RA control mice, the immunoreactivity of CBS was mainly localized to the ganglion cell layer (GCL), inner plexiform layer (IPL), and inner nuclear layer (INL), and was weakly detected in the outer plexiform layer (OPL). In OIR retina, there was increased CBS immunoreactivity in all of these layers (Fig. 3A). More strikingly, CBS staining was prominently increased in the outer plexiform layer (OPL). In OIR retina, there was increased CBS immunoreactivity in all of these layers (Fig. 3A). More strikingly, CBS staining was prominently increased in the outer plexiform layer (OPL). In OIR retina, there was increased CBS immunoreactivity in all of these layers (Fig. 3A). More strikingly, CBS staining was prominently increased in the outer plexiform layer (OPL). In OIR retina, there was increased CBS immunoreactivity in all of these layers (Fig. 3A). 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More interestingly, CSE staining was also identified in some large cells with the morphology of leukocytes within NV tufts, consistent with other reports that CSE is a component of leukocytes.

**Pharmacologic Inhibition of CBS/CSE Reduces Retinal Neovascularization**

The upregulation of H\textsubscript{2}S production and H\textsubscript{2}S-producing enzymes CBS and CSE in OIR suggests a potential involvement of this pathway in retinal NV. To test this possibility, we
pharmacologically inhibited CBS and CSE with aminooxyacetic acid (AOAA), which has classically been considered a selective CBS inhibitor but has also been shown to significantly inhibit CSE.29 Injection of AOAA (IP 3 mg/kg) resulted in moderate (~25%) inhibition of H2S production in vivo (Supplementary Fig. S2). To study the effect of CBS/CSE inhibition on retinal NV, AOAA was injected daily from P12 to P16, during the relative hypoxic phase of OIR. Treatment with AOAA did not affect body weight (Fig. 4A), but significantly reduced the amount of retinal NV, from 13.4% in control mice to 9.5% in AOAA-treated littermates (Fig. 4B). These findings indicate that (CBS and CSE)/H2S pathway is involved in the pathogenesis of ischemia-induced retinopathy.

Inhibition of CBS and CSE Does Not Change the Expression of Hypoxia-Driven Angiogenic Molecules in Ischemic Retina

Hypoxia-inducible factor 1 (HIF-1α)-mediated upregulation of angiogenic molecules such as VEGF and EPO has an essential role in pathologic NV in ischemia-induced retinopathy.39-41 Disruption of HIF-1α in Müller cells reduces VEGF production in the retina and significantly alleviates NV in OIR.32 Since H2S has been shown to promote hypoxia-induced VEGF expression by enhancing HIF-1α expression and activity,14,45-46 we determined whether H2S was involved in the pathologic NV in OIR by regulating HIF-1α-mediated production of angiogenic molecules.14,45-46 We observed that CBS and CSE inhibition by AOAA did not affect the expression of VEGF, EPO, and angiopoietin-2 in OIR (Fig. 5), suggesting that (CBS and CSE)/H2S regulates retinal NV via a mechanism independent of HIF-1α-mediated expression of these angiogenic molecules in ischemia-induced retinopathy.

CSE Contributes to Retinal Neovascularization in Oxygen-Induced Retinopathy

Cystathionine-γ-lyase is the main H2S-producing enzyme in the vasculature.22,23 Blocking CSE attenuates VEGF signaling in endothelial cells and markedly reduces microvessel formation in response to VEGF.17 The prominent increase of CSE expression in NV tufts suggests that the CSE/H2S pathway may have a role in retinal NV during ischemia-induced retinopathy. Therefore we generated littermate mice with wild-type (CSE+/+) and heterozygous CSE alleles (CSE+/−), or littermate mice with heterozygous (CSE+/−) and homozygous (CSE−/−) CSE deletion, and confirmed CSE deficiency in the retina (Supplementary Fig. S3A). Compared with CSE+/+ mice, H2S production in the plasma of CSE−/− mice was reduced by ~50% (Supplementary Fig. S3B), which is consistent with the study of Yang et al.13 After examination of retinal vasculature in

**FIGURE 3.** The expression of CBS and CSE is increased in the retinas of OIR. Retinal frozen sections from P17 RA or OIR mice were stained with antibodies for (A) CBS (red) and GFAP (green) or (B) CSE (green) and isolectin B4 (red). Nuclei were stained with DAPI (blue). Representative images were taken with fluorescence microscopy at ×200 magnification. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.
the OIR model, we observed that CSE−/− retinas displayed phenotypes identical to those of CSE+/− retinas. There was no difference in vascular dropout area and NV tuft area between CSE+/− and CSE−/− mice (Supplementary Fig. S4). However, CSE−/− OIR mice exhibited a 35% reduction of neovascular area compared with their CSE+− OIR littermates at P17 (Fig. 6A), although the two genotypes had similar avascular area at P12 after exposure to hyperoxia (Fig. 6B). These data suggest that CSE-derived H2S contributes to pathologic retinal NV during ischemia-induced retinopathy. CSE−/− OIR mice also had slightly increased avascular area at P17 compared with CSE+/− OIR mice (Fig. 6A).

**DISCUSSION**

Hydrogen sulfide is a member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO). It has been shown that H2S can promote angiogenesis in organs including the heart, hindlimb, and tumor; however, the role of H2S in retinal NV is yet unclear. Here we demonstrate that H2S-generating enzymes (CBS and CSE) and H2S production are increased in the retinas from OIR mice, and that blocking CBS and CSE significantly reduces retinal NV during ischemia-induced retinopathy. Given that H2S induces angiogenesis via VEGF-dependent and VEGF-independent mechanisms, our data suggest that blockade of H2S-generating enzymes may provide an alternative therapeutic approach to prevent vision loss due to retinal NV in ischemia-induced retinopathy, even when anti-VEGF agents are not effective. Moreover, CBS and CSE inhibition may offer some advantages since it specifically reduces retinal pathologic NV but has minimal negative effect on vascular repair, unlike VEGF trap, which reduces retinal NV but blocks retinal revascularization and increases avascular area.

As a reducing agent and an antioxidant molecule that modulates many downstream signaling events, H2S has been widely known to protect various cell types under stress/pathologic conditions. In the central nervous system, H2S protects neuronal cell damage in stroke, traumatic brain injury, and spinal cord injury via multiple actions including dilation of cerebral vessels, inhibition of neuronal inflammation, prevention of activation of apoptotic pathways, and counteraction of glutamate-mediated excitotoxicity. In the cardiovascular system, H2S prevents against atherosclerosis, promotes vasorelaxation, and preserves endothelial mitochondrial function during hyperglycemia. In the retina, H2S attenuates excitotoxic neurotransmission, prevents light-induced excitotoxicity, reduces neuronal injury after ischemia/reperfusion, and alleviates retinal oxidative stress and inflammation in diabetes. While plenty of evidence indicates that promoting H2S production is beneficial, pathologic roles of H2S are also suggested. Hydrogen sulfide may participate in
secondary neuronal injury of many CNS diseases by promoting calcium overload. Upregulation of the CBS–H2S pathway has been found to promote the development of neuropathic pain and tumorigenesis. Our study showing that the CBS/CSE-derived H2S is involved in retinal NV during ischemia-induced retinopathy represents the first case in which overactivating H2S was harmful in the retina. Overall, H2S exhibits complex roles depending on the cellular and disease contexts. Consequently, while our study suggests that blocking H2S production at the proliferative stage of ischemia-induced retinopathy can be beneficial, cautions are warranted when using this strategy. Reduction of H2S production at an early stage of ischemia-retinopathy might lessen its neuronal and vascular protective effects and therefore accelerate the progression of disease. Additionally, local inhibition of H2S-generating enzymes is preferred since systemic blockade of H2S may cause impairment of cardiovascular functions.

Three H2S-generating enzymes (CBS, CSE, and 3-MST) have been identified, all of which are expressed in the retina. The expression of CBS and CSE is upregulated in OIR, suggesting that these two enzymes are implicated in pathologic changes in OIR. In contrast to CBS and CSE, the level of 3-MST is not changed. By inhibiting H2S production with a pharmacologic inhibitor for both CBS and CSE or genetic deletion of CSE, we showed that H2S is a novel player in retinal NV. Hydrogen sulfide is known to induce angiogenesis by inducing endothelial cell proliferation, migration, and tube formation. During OIR, H2S overproduction by upregulation of CSE in neovascular tufts or CBS in glia and neurons may promote retinal pathophysiological angiogenesis by modifying the angiogenic abilities of endothelial cells. Mechanistically, H2S may activate a variety of pathways including Akt activation, synergistic interaction with nitric oxide–induced angiogenic response, sulfhydration and activation of KATP channels, upregulation of VEGF receptor (VEGFR)2 and neuropilin (NRP)-1 via stabilization of the transcription factor specificity protein and VEGFR2 activation by reducing an inhibitory disulfide bond in its intracellular region that disrupts signal transduction. In addition to its direct effect to stimulate the angiogenic ability of endothelial cells, H2S-mediated retinal NV may indirectly involve its actions on other retinal cell types. Since H2S has been shown to promote hypoxia-induced VEGF expression by enhancing HIF-1 expression and activity and CBS is upregulated in multiple retinal cell types, we reasoned that upregulation of H2S may enhance VEGF expression in OIR. We measured several HIF-1-driven angiogenic molecules including VEGF in OIR retinas, but CBS/CSE inhibitor did not reduce the expression of these molecules. Future studies comparing retinal gene expression profiles between vehicle and CBS/CSE inhibitor-treated OIR retinas are needed to identify its potential downstream targets.

In summary, our data provide the first evidence that H2S-generating enzymes/H2S pathway is activated and involved in pathologic retinal NV during ischemia-induced retinopathy. The limitation of the study is that we were not able to specify the role of CBS in retinal NV because CBS-deficient mice exhibit retinal ischemia and vascular defects during development, which precludes the use of these mice to study pathologic changes in OIR, and because there is no selective pharmacologic CBS inhibitor. Further studies conditionally deleting CBS in retinal neurons and glia will be needed to explore the precise contribution of CBS-derived H2S in retinal NV. Since NV also occurs in age-related macular degeneration, neovascular glaucoma, and corneal injury, our study calls for further investigation of the H2S pathways to determine if targeting this pathway is beneficial in the treatment of a variety of diseases.
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