

Hydrogen Sulfide: A Potential Therapeutic Target in the Development of Diabetic Retinopathy

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PURPOSE. Hyperglycemia damages the retinal mitochondria, and the mitochondrial damage plays a central role in the development of diabetic retinopathy. Patients with diabetes also have higher homocysteine levels, and abnormalities in homocysteine metabolism result in decreased levels of hydrogen sulfide (H₂S), an endogenous gas-transmitter signaling molecule with antioxidant properties. This study aimed to investigate the role of H₂S in the development of diabetic retinopathy.

METHODS. Streptozotocin-induced diabetic mice were administered a slow releasing H₂S donor GYY4137 for 6 months. The retina was used to measure H₂S levels, and their retinal vasculature was analyzed for the histopathology characteristic of diabetic retinopathy and oxidative stress, mitochondrial damaging matrix metalloproteinase-9 (MMP-9), and mitochondrial integrity. These parameters were also measured in the isolated retinal endothelial cells incubated in high glucose medium containing GYY4137.

RESULTS. Administration of GYY4137 to diabetic mice ameliorated decrease in H₂S and prevented the development of histopathology, characteristic of diabetic retinopathy. Diabetes-induced increase in oxidative stress, MMP-9 activation, and mitochondrial damage were also attenuated in mice receiving GYY4137. Results from isolated retinal endothelial cells confirmed the results obtained from diabetic mice.

CONCLUSIONS. Thus, supplementation of H₂S donor prevents the development of diabetic retinopathy by ameliorating increase in oxidative stress and preserving the mitochondrial integrity. H₂S donors may provide a novel therapeutic strategy to inhibit the development of diabetic retinopathy.

Keywords: antioxidant, diabetic retinopathy, homocysteine, hydrogen sulfide, mitochondria, MMP-9

Diabetic retinopathy is the leading cause of acquired blindness, and despite extensive research in the field, its etiology remains elusive. In the early stage of diabetic retinopathy, retinal capillary cells undergo accelerated apoptosis resulting in pericyte ghosts and acellular capillaries.¹⁻³ A growing body of evidence supports the hypothesis that oxidative stress mitochondrial damage contributes to accelerated apoptosis of retinal capillary cells. Reactive oxygen species (ROS), by damaging the mitochondrial membranes, alter their potential, and allow cytochrome c to leak out into the cytosol, leading to cell apoptosis.^{4,5} One of the molecular consequences of oxidative stress is the activation of matrix metalloproteinases (MMPs); ROS oxidize sulfide bond in their pro-domains, and also reduce their tissue inhibitors of MMPs (TIMPs).^{6,7} Diabetes transcriptionally and functionally activates gelatinase metalloproteinase-9 (MMP-9), and suppresses its tissue inhibitor Timp1 in the retina and its capillary cells.^{3,8,9} In the early stages of diabetic retinopathy, activated gelatinase MMPs act as apoptotic inducer, and damages the mitochondria. Our previous study has documented a direct role of MMP-9 in the development of diabetic retinopathy, and have shown that diabetic mice with the MMP-9 gene knocked out are protected from the development of histopathology characteristic of diabetic retinopathy.^{3,7}

Patients with diabetes also have elevated levels of homocysteine, a nonproteinogenic, sulfur-containing amino acid, and elevated homocysteine is now recognized as an independent risk factor of diabetic retinopathy.^{10,11} Homocysteine is metabolized to cysteine by transsulfuration process using cystathionine-β synthase (CBS) and cystathionine γ-lyase (CSE), and cysteine is also a substrate for CBS and CSE to generate a gasotransmitter hydrogen sulfide (H₂S).^{12,13} An imbalance between homocysteine and H₂S increases oxidative stress, inflammation, and ischemic injury.^{13,14} H₂S is generally considered as a toxic gas, which can affect the central nervous system, but recently it is also recognized as a signaling molecule with significant cytoprotective effects.¹⁵ Our recent study has shown that retina from human donors with established diabetic retinopathy although have increased homocysteine compared to their age-matched nondiabetic human donors, H₂S levels in the same diabetic donors is significantly lower.¹⁶ Accumulating evidence has suggested various physiological functions of H₂S, including neuromodulation, vasodilation, inflammation, and apoptosis.¹⁷⁻¹⁹ However, the role of H₂S in diabetic retinopathy remains in its incipient stages.

The goal of this study is to investigate the role of H₂S in diabetic retinopathy, focusing on its role in increasing oxidative stress MMP-9 activation. Using retinal

TABLE 1. Effect of GY on Body Weight and Glucose of Diabetic Mice

Group	Body Weight (g) Initial → Final	Blood Glucose (mg/dl) Initial → Final
Normal	18.9 ± 1.5 → 36 ± 4.7	130 ± 17 → 124 ± 21
Diabetes	19.8 ± 1.8 → 25 ± 3.1*	378 ± 97* → 457 ± 85*
Diabetes + GY	20.2 ± 1.4 → 24 ± 4.1*	378 ± 108* → 463 ± 112*
Diabetes + homocysteine	20.7 ± 1.7 → 26 ± 3.7*	366 ± 106* → 419 ± 108*
Diabetes + homocysteine + GY	19.6 ± 2.1 → 25 ± 2.3*	405 ± 117* → 434 ± 118*

Each group had 10 or more mice, and body weight and blood glucose are the mean ± SD of the values obtained in the beginning (initial) and at the end of the experiment (final, 6 months).

* $P < 0.05$ versus normal.

microvessels from streptozotocin-induced diabetic mice, receiving a slow-releasing pharmacological donor of H₂S, we have investigated the effect of regulation of H₂S on ROS-MMP-9-mitochondrial damage. The in vivo results were confirmed in the human retinal endothelial cells (HRECs), exposed to high glucose.

METHODS

Mice

Diabetes was induced in 7 to 9-week-old C57BL/6J mice (both male and female; Jackson Laboratory, Bar Harbor, ME, USA) by administration streptozotocin (55 mg/kg BW, intraperitoneal) for four consecutive days between 4 PM and 5 PM. The mice presenting blood glucose > 250 mg/dL, 2 days after the last injection, were considered diabetic. We routinely induce hyperglycemia by streptozotocin administration in both male and female mice, and can maintain similar severity of hyperglycemia by adjusting their insulin regimen.^{3,20,21} Diabetic mice were divided into 4 groups, group 1 mice received a slow-releasing H₂S donor GYY4137 (GY; Cat. No. SML0100 Sigma-Aldrich, St. Louis, MO, USA; DMSO solution diluted in normal saline, 0.25 mg/Kg, intraperitoneal)²² every other day (Diab/GY); group 2 mice received intraperitoneally 100 mg/kg BW DL-homocysteine (Cat. No. 44925; Sigma-Aldrich),²³ dissolved in PBS (Diab/H), and group 3 mice received both of DL-homocysteine and GY (Diab/H + GY). Mice in group 4 remained diabetic, without any supplementation (Diab); each group had a total of 10 to 15 mice. Supplementation of GY, homocysteine, or homocysteine and GY was initiated soon after the establishment of hyperglycemia (2 days after the last streptozotocin injection). Neither GY or homocysteine had any effect on the body weight and blood glucose of these diabetic mice, and the values obtained at the initiation, and at 6 months were similar in all of the four groups of diabetic mice ($P < 0.05$ versus normal and $P > 0.05$ versus diabetes; Table 1). Six months after induction of diabetes, the animals were euthanized, one eye was immediately fixed in 4% paraformaldehyde for histopathology, and the other eye was used to isolate the retina. Age-matched normal mice (male and female) were used as controls (Norm), and each study had retina from similar numbers of male and female mice. The treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Wayne State University's Institutional Animal Care and Use Committee.

Microvessels were prepared by incubating the retina at 37°C in 5 to 6 mL deionized water for 60 minutes in a shaking water bath. The nonvascular tissue was gently removed under a dissecting microscope using a Pasteur pipette, and

the microvasculature was largely (> 95%) devoid of nonvascular tissue.¹⁶

Retinal Endothelial Cells

Primary human retinal endothelial cells (HRECs) were purchased from Cell Systems Corporation (Cat. No. ACBRI 181; Cell Systems Corp., Kirkland, WA, USA), and were cultured in Dulbecco's modified Eagle medium (DMEM)-F12, as described previously.^{23,24} Cells from the sixth to ninth passage, with 80 to 90% confluency, were incubated in medium containing normal glucose (NG; 5 mM D-glucose) or high glucose (HG; 20 mM D-glucose) for 96 hours in the presence or absence of 100 μM L-homocysteine thiolactone hydrochloride (HG/H; Cat. No. S784036; Sigma-Aldrich). To investigate the effect of regulation of H₂S, 150 μM GY²² was supplemented in high glucose (HG/GY), and in high glucose + homocysteine (HG/H + GY) incubations. To rule out the effect of osmolarity, each experiment included HRECs incubated in 20 mM L-glucose (L-Glu), instead of 20 mM D-glucose.

Hydrogen Sulfide

H₂S was measured in the cell media or retinal homogenate using methylene blue assay, as previously reported.¹⁶ Briefly, to trap H₂S, 350 μl of the incubation media or 30 μg retinal homogenate was transferred directly into a cryovial tube containing 1% (w/v) zinc acetate. Following incubation for 10 to 20 minutes at room temperature, 20 mM N-dimethyl-p-phenylenediamine sulfate (in 7.2 M HCl) and 30 mM FeCl₃ (in 1.2 M HCl) were added, and the mixture was incubated in dark at 37°C (retina = 1–2 hours; culture media = 15–30 minutes). Protein was removed by 10% trichloroacetic acid, and the absorbance in the supernatant was measured at 670 nm, using sodium hydrosulfide (NaHS) as a standard.

Endogenous H₂S in cells was measured by in situ fluorescence microscopy using the fluorescence probe azido-4-methylcoumarin.²⁵ HRECs grown on coverslips were incubated with 50 μM 7-azido-4-methylcoumarin (Cat. No. 802409; Sigma-Aldrich) for 30 minutes, washed with PBS, and the fluorescence response of 7-Azido-4-Methylcoumarin to H₂S was visualized using Zeiss ApoTome fluorescence microscope (Carles Zeiss Inc., Chicago, IL, USA) at 20 times magnification.

Homocysteine

Homocysteine levels were quantified in 25 μg retinal protein using an ELISA kit from Cell Bio Labs Inc. (Cat. No.

TABLE 2. Primer Sequences

Primer	Size (bp)	Sequence
Mouse		
<i>mtDNA-short</i>	117	Fwd-CTA GCA GAA ACA AAC CGG GC Rev-CCG GCT GCG TAT TCT ACG TT
<i>mtDNA-long</i>	10,090	Fwd-GCC AGC CTG ACC CAT AGC CAT AAT AT Rev- GAG AGA TTT TAT GGG TGT AAT GCG G
<i>Cytb</i>	75	Fwd-AGA CAA AGC CAC CTT GAC CCG AT Rev-ACG ATT GCT AGG GCC GCG AT
<i>ND6</i>	173	Fwd-CCC AGC TAC TAC CAT CAT TCA AGT Rev-GAT GGT TTG GGA GAT TGG TTG ATG T
<i>18S</i>	149	Fwd-GCC CTG TAA TTG GAA TGA GTC CAC TT Rev-CTC CCC AAG ATC CAA CTA CGA GCT TT
Human		
<i>mtDNA-short</i>	223	Fwd-CCC CAC AAA CCC CAT TAC TAA ACC CA Rev-TTT CAT CAT GCG GAG ATG TTG GAT GG
<i>mtDNA-long</i>	8,842	Fwd-TCT AAG CCT CCT TAT TCG AGC CGA Rev-TTT CAT CAT GCG GAG ATG TTG GAT GG
<i>Cyt b</i>	138	Fwd-TCA CCA GAC GCC TCA ACC GC Rev-GCC TCG CCC GAT GTG TAG GA
<i>β-Actin</i>	237	Fwd-AGC CTC GCC TTT GCC GAT CCG Rev-TCT CTT GCT CTG GGC CTC GTC G

STA-670; San Diego, CA, USA), as described previously.²³ Homocysteine-BSA was used as a standard, and homocysteine content was normalized to the total protein in the sample.

Activities of CBS and CSE

CBS activity was measured using 40 to 60 µg retinal protein using cystathionine β synthase activity assay kit (Cat. No. K998; Bio Vision, Milpitas, CA, USA) and cysteine and homocysteine as substrates, as reported previously.¹⁶ The specificity of CBS activity was measured by using 7-amino-4-methylcoumarin as a standard.

CSE activity was measured according to the published methods.²⁶ Briefly, approximately 50 µg protein was incubated with 10 mM L-cysteine and the cofactor pyridoxal-5'-phosphate (2 mM) at 37°C in a shaking water bath for 2 hours. The reaction mixture was then transferred directly into a cryovial tube containing 1% (w/v) zinc acetate, and 20 mM N-dimethyl-p-phenylenediamine sulfate (in 7.2 M HCl) and 30 mM FeCl₃ (in 1.2 M HCl) were then added. After incubating for 15 to 30 minutes at 37°C in the dark, the proteins were precipitated by 10% trichloroacetic acid, and absorbance of the resulting supernatant was measured at 670 nm. The amount of H₂S produced/mg protein/hour was calculated.

Histopathology

Whole retina was carefully isolated from the paraformaldehyde fixed eye, and incubated at 37°C for 45 to 75 minutes in 3% crude trypsin (Gibco, Grand Island, NY, USA) solution containing 200 mM sodium fluoride. The neuroretinal tissue was gently brushed away under a microscope, and the vasculature was stained with periodic acid Schiff-hematoxylin to count the acellular capillaries by light microscopy, and imaged using the Zeiss ApoTome microscope using a 40× objective.^{3,20}

Vascular Permeability and Optical Coherence Tomography

Micron IV (Phoenix Research Labs, Pleasanton, CA, USA) retinal imaging system was used to quantify retinal thickness. After anesthetizing mice with Ketamine-Xylazine, their pupils were dilated using 1% tropicamide ophthalmic solution, and the cornea was lubricated with Goniovisc. After photographing the fundus using fundus camera for small animals, the animals were injected with AK-FLUOR (0.5% solution, 0.01 mL/g BW, intraperitoneally), and their fundus was photographed 10 minutes after fluorescein injection using a barrier filter for fluorescein angiography.²⁰ The images were converted into 8 bit using imageJ 1.53a and the threshold was set equally in each group of mice with the setting adjusted to dark background. Zeiss imaging software analysis module was used to mark the vessels "Red," and the leakage was quantified by region of interest in the visible white area.²⁷ The value obtained from normal mice was considered as 100%.

For optical coherence tomography (OCT), after lubricating the pupils with Goniovisc, the eyes were positioned in front of the OCT camera to obtain high-resolution b-scan of the retinal cross-sections, as described previously.²⁰ Thickness of total retinal layers, ganglion cell layer + inner plexiform layer (GCL + IPL) and of inner nuclear layer (INL) was measured at 200 to 400 µm distance on either side of the optic disc using the caliper tool in the InSight software.

Immunofluorescence Staining

Retinal cryosections (8 µm) were incubated with homocysteine or MMP-9 antibodies (Cat. No. ab15154 and Cat. No. ab119906, respectively; Abcam, Cambridge, MA, USA; each at 1:200 dilution). Fluorescence labeled secondary antibodies included DyLight-488 labeled (green) for homocysteine and Texas Red (red) for MMP-9 (Cat. Nos. DI-1488 and TI200, respectively, Vector Laboratories, Burlingame, CA, USA; each at 1:500 dilution). The sections mounted with DAPI (blue) containing medium were photographed by ZEISS ApoTome fluorescence microscope using a 20× objective.²⁰

Reactive Oxygen Species

Total ROS were quantified using 2',7'-dichlorofluorescein diacetate (DCFDA; Cat. No. D6883; Sigma-Aldrich) method in cell lysate or retinal microvessel (5 μ g protein).²¹ Percentage change was calculated considering the values obtained from cells in normal mice, or normal glucose, as 100%.

Mitochondrial ROS were quantified using MitoSOX Red (Cat. No. M36008; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the nonadherent cells were removed by washing with DMEM, and incubated with 5 μ M MitoSOX red for 20 minutes at 37°C.²⁴ The fluorescence was visualized using Zeiss ApoTome fluorescence microscope using a 20 \times objective.

Protein Carbonyls

Protein carbonyls were quantified in 15 to 25 μ g protein using 2,4-dinitrophenylhydrazine derivatization, according to the manufacturer's instructions (Protein Carbonyl Assay Kit, Cat. No. ab126287; Abcam, Cambridge, MA, USA).

Protein carbonyls concentration was calculated using BSA as a standard.²⁸

MMP-9 Activity

Enzyme activity was quantified by fluorescence kit using a specific anti-MMP-9 monoclonal antibody and a fluorogenic substrate (Sensolyte Plus 520 MMP-9 Assay Kit; AnaSpec, Inc., Fremont, CA, USA), as described previously.²⁹ MMP-9 induced cleavage of the fluorogenic peptide was measured at 490 nm excitation and 520 nm emission wavelengths.

Gene Transcripts

RNA extracted by TRIZOL (Invitrogen, Carlsbad, CA, USA) was used to prepare cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using SYBR green master mix (Applied Biosystems) and the gene- and species-specific primers (Table 2). Specific products were confirmed by SYBR green single melt curve analysis.^{21,23} Housekeeping genes included 18S rRNA

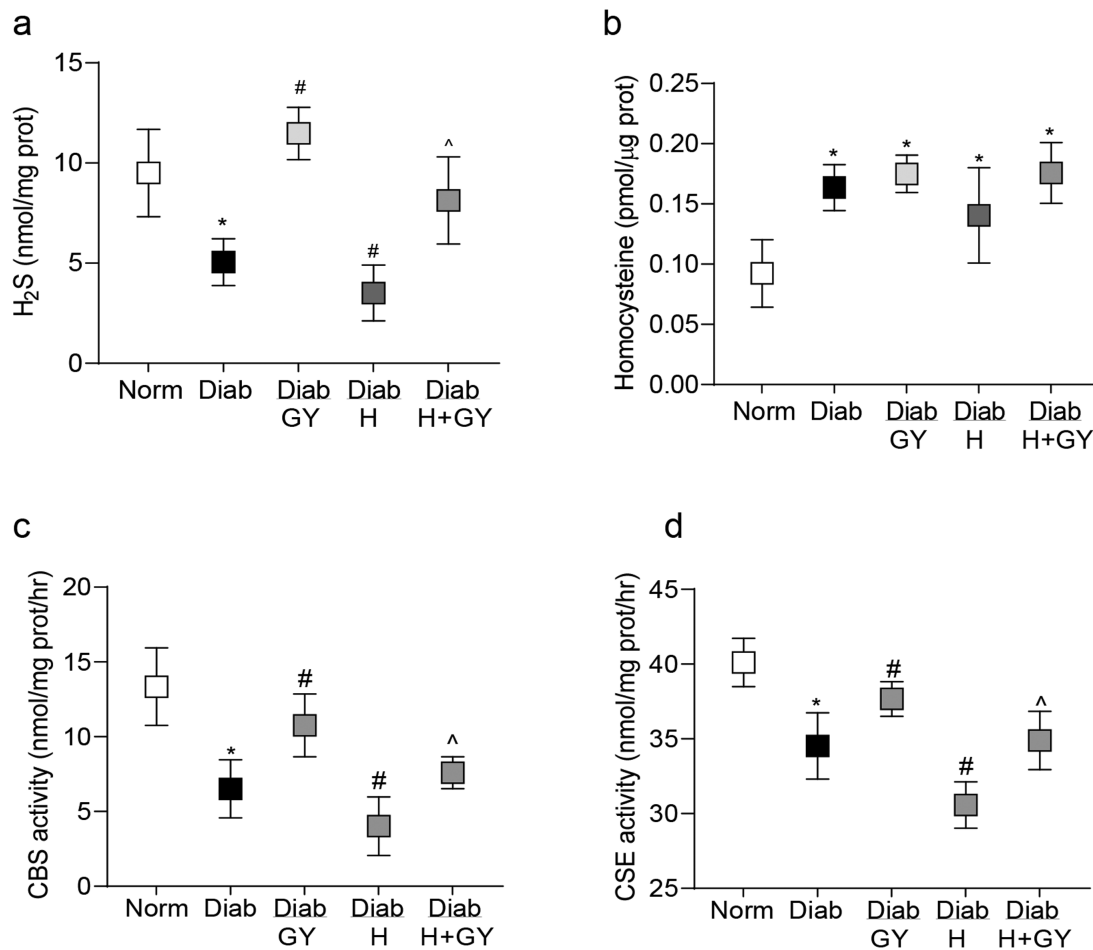


FIGURE 1. Effect of hydrogen sulfide donor on diabetes-induced alterations in H₂S and its enzymatic machinery. Retina from diabetic mice, with or without homocysteine and GY supplementation, were analyzed for (a) H₂S and (b) homocysteine levels using their respective colorimetric methods. (c) CBS activity was quantified using cysteine and homocysteine as substrates, and (d) CSE activity using L-cysteine as a substrate and pyridoxal-5'-phosphate as a cofactor. Each measurement was made in duplicate in 5 to 7 mice/group, and the values are represented as mean \pm SD. Norm, normal; Diab and Diab/GY, diabetic mice without or with GY respectively; Diab/H and Diab/H + GY, homocysteine supplemented diabetic mice, without or with GY. **P* < 0.05 versus normal, #*P* < 0.05 versus diabetes, and ^*P* < 0.05 versus diabetic mice receiving homocysteine.

for mice and β -actin for HRECs. Relative fold change was calculated using the delta-delta Ct method.

Mitochondrial DNA Damage

Damage of mitochondrial DNA (mtDNA) was quantified by extended length PCR, as detailed previously.³⁰ In brief, long and short mtDNA regions (10.0 kb and 117 bp for mice and 8.8 kb and 223 bp for HRECs) were amplified using semi-quantitative PCR, and the amplified products were resolved on an agarose gel. Relative amplification was quantified by

normalizing the intensity of the long product to the short product; the degree of the damage was inversely proportional to the ratio.

Mitochondrial Membrane Potential

Changes of mitochondrial membrane potential were measured by staining cells with a mitochondrial binding dye, JC-1 (Cat. No. MP03168, Molecular Probes, Carlsbad, CA, USA). As reported previously,³¹ the cells were washed with PBS and incubated with DMEM containing 5 μ M JC-1 for

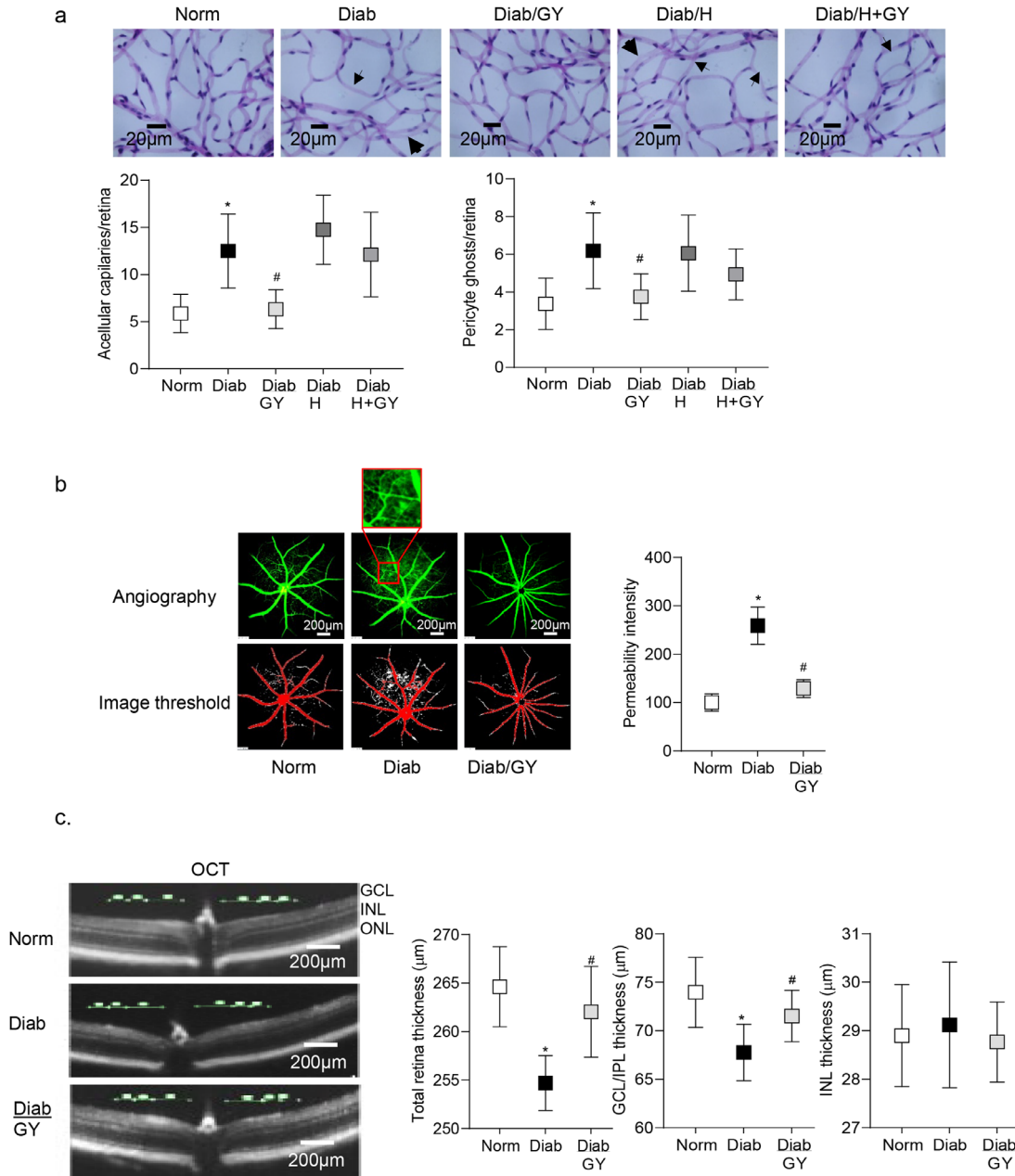


FIGURE 2. Hydrogen sulfide donor and retinal damage in diabetic mice. Representative images from 5 to 7 mice/group showing (a) trypsin-digested retinal microvessels, stained with PAS showing acellular capillaries (*thin arrow*) and pericyte ghosts (*thick arrowhead*); (b) fluorescein angiography. The insert shows vascular leakage, and the histogram represents permeability intensity (c) OCT by the OCT module of Micron IV showing thickness of the total retinal layers and GCL + IPL at 200 to 400 μ m away from the optic disc using the caliper tool in the InSight software; the histograms represent mean \pm SD of the values from those mice at 300 μ m. * $P < 0.05$ and # $P < 0.05$ compared to normal and diabetes, respectively.

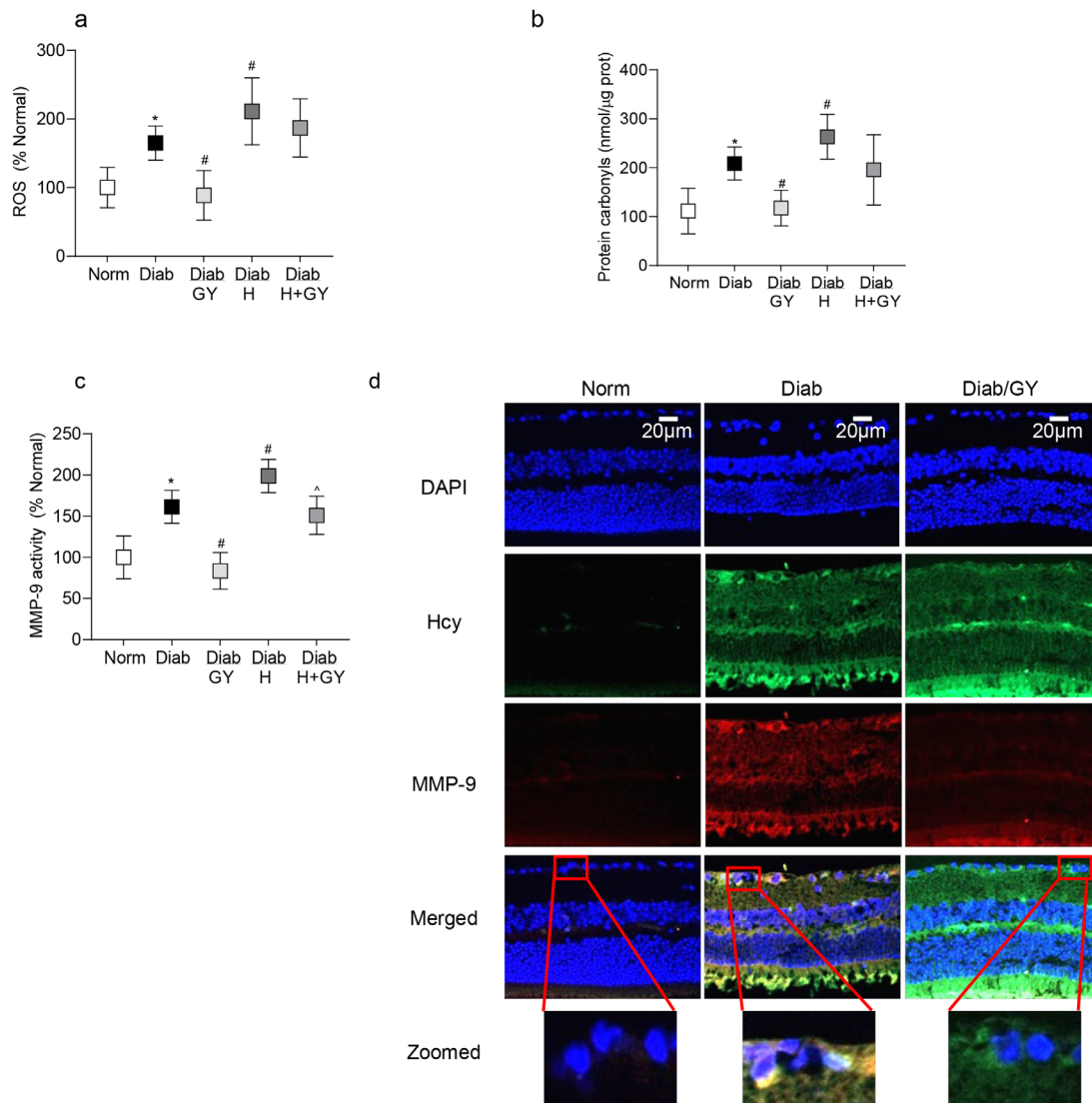


FIGURE 3. GY supplementation and oxidative stress and MMP-9 activation. Mice retinal microvessels prepared by osmotic shock were analyzed for oxidative stress by quantifying (a) ROS levels using DCFDA and (b) protein carbonyls employing 2,4-dinitrophenylhydrazine. (c) MMP-9 activity was measured using its monoclonal antibody and a fluorogenic substrate. (d) Representative image of the co-localization of homocysteine and MMP-9 using DyLight-488 (green) and Texas Red (red) conjugated secondary antibodies for homocysteine and MMP-9, respectively. Each measurement was made in duplicate in 6 to 7 mice/group, and the values are represented as mean \pm SD. Norm, normal; Diab and Diab/GY, diabetic mice without or with GY respectively; Diab/H and Diab/H + GY, homocysteine supplemented diabetic mice, without or with GY. * $P < 0.05$ versus normal, # $P < 0.05$ versus diabetes, and ^ $P < 0.05$ versus diabetic mice receiving homocysteine.

30 minutes at 37°C. Cells were again washed with PBS, and visualized under Zeiss ApoTome fluorescence microscope at 20 times magnification.

Cell Apoptosis

Cell death was measured by a photometric enzyme immunoassay using the Cell Death Detection ELISA PLUS kit from Roche Diagnostics (Cat. No. 11774425001; Indianapolis, IN, USA), as described previously.²³ The values obtained from cells in normal glucose were considered as 100%.

Statistical Analysis

Data are presented as mean \pm standard deviation (SD). SigmaStat software (Systat, San Jose, CA, USA) was used

to perform statistical analyses, and comparison between groups was made using 1-way ANOVA followed by a post hoc Bonferroni test; $P < 0.05$ was considered significant.

RESULTS

Diabetic Mice

In accordance with the results from human disease samples,¹⁶ retina from diabetic mice had approximately 45% less H₂S and approximately 2 fold higher homocysteine compared to their age-matched normal mice (both, $P < 0.05$ versus normal group; Figs. 1a, 1b). Because both CBS and CSE are involved in the transsulfuration process to produce H₂S,^{12,13} their enzyme activities were quantified. As

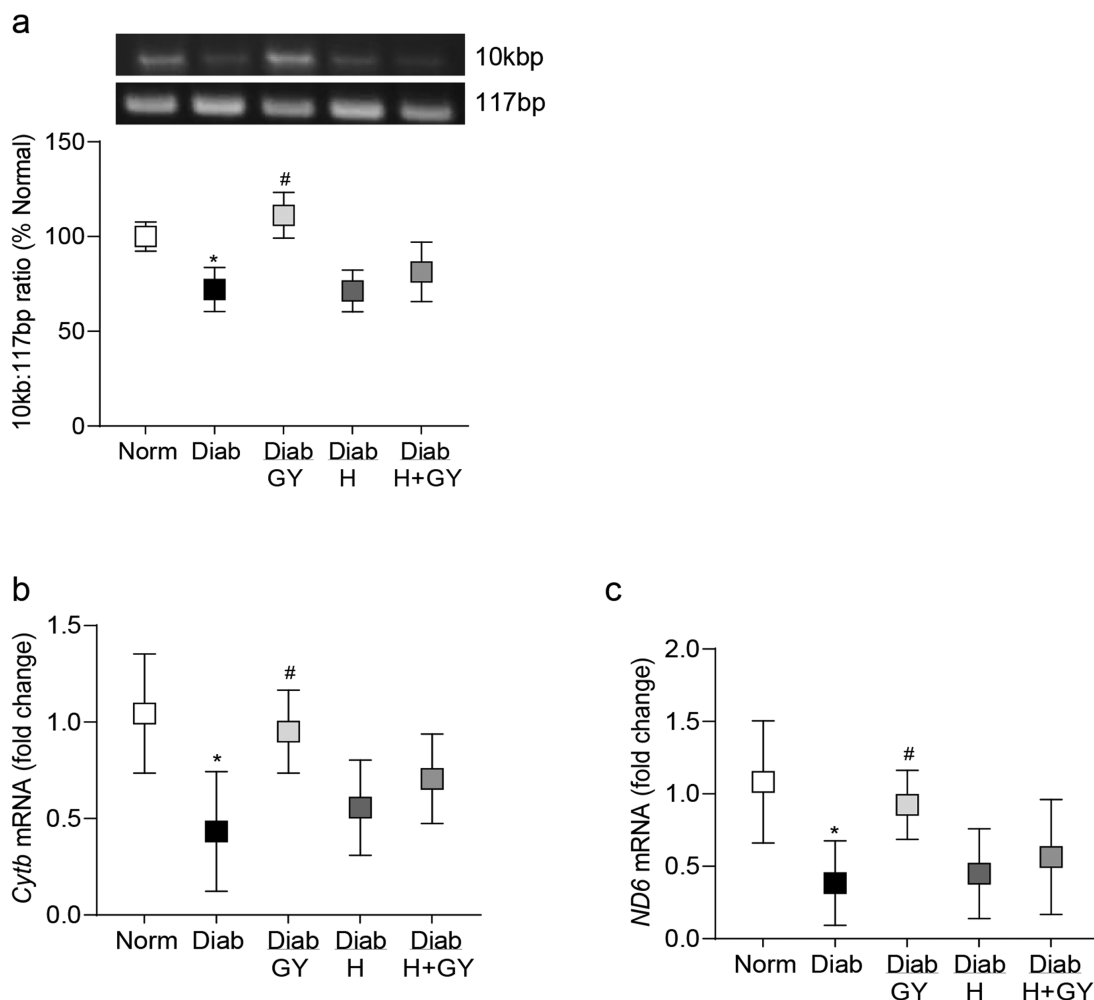


FIGURE 4. Mitochondrial damage and GY supplementation. Mouse retinal microvessels were employed to quantify (a) mtDNA damage by extended length PCR by quantifying relative amplification of 10 kb and 117 bp products; lower ratio represents more damage. The gene transcripts of (b) *Cytb* and (c) *ND6* were quantified by real time qRT-PCR using 18sRNA as a housekeeping gene. Values are represented as mean \pm SD from six mice/group, with each measurement made in duplicate. * $P < 0.05$ versus normal and # $P < 0.05$ vs. diabetes.

shown in Figures 1c, 1d, both CBS and CSE were decreased significantly in the retina from diabetic mice. Administration of GY soon after induction of diabetes (Diab/GY group), although failed to attenuate increase in retinal homocysteine, it ameliorated decrease in H_2S content and the activities of CBS and CSE. H_2S levels and the enzyme activities in diabetic mice receiving GY were significantly higher compared to diabetic mice without GY supplementation ($P < 0.05$ versus Diab group; Figs. 1a–d).

The effect of restoration of H_2S on the development of diabetic retinopathy was determined by quantifying the numbers of degenerative capillaries and pericyte ghosts in the trypsin digested retina. As shown in Figure 2a, diabetes-induced increase in the number of degenerative capillaries and pericyte ghosts was prevented in Diab/GY group, and the values from mice in the Norm and Diab/GY groups were not different from each other ($P > 0.05$ versus normal and $P < 0.05$ versus Diab group).

The effect of GY administration on vascular integrity was further confirmed by fluorescence angiography. Compared with normal control mice, diabetic mice had significant leakage of fluorescein in the retinal vasculature 10 minutes after its administration. However, GY administration signif-

icantly attenuated vascular permeability in diabetic mice (Fig. 2b). Fundus OCT data from the same mice receiving GY showed amelioration of diabetes-induced thinning of the retina and of GCL + IPL ($P < 0.05$ versus Diab group; Fig. 2c), but compared with normal mice, thickness of INL was not changed observed in diabetic mice, and GY had no effect on this layer.

H_2S is considered to act as an antioxidant^{13,14}; the effect of GY on oxidative stress was determined in retinal microvasculature. GY administration ameliorated diabetes-induced increase in ROS and protein carbonyls; the values in Diab/GY group were significantly lower than those in the Diab group ($P < 0.05$ versus Diab group; Figs. 3a, 3b). Increase in ROS is shown to contribute to the activation of redox-sensitive MMP-9^{8,32}; GY administration ameliorated MMP-9 activation ($P < 0.05$ versus Diab group), and the colocalization of MMP-9 and homocysteine in retinal microvasculature was also decreased (Figs. 3c, 3d).

Activation of MMP-9 is implicated in the mitochondrial damage^{3,33}; the effect of GY on retinal mitochondrial damage was investigated. Compared to diabetic mice, GY receiving diabetic mice had significantly lower ratio of amplification of 10 kb/117 bp amplicons ($P < 0.05$ versus

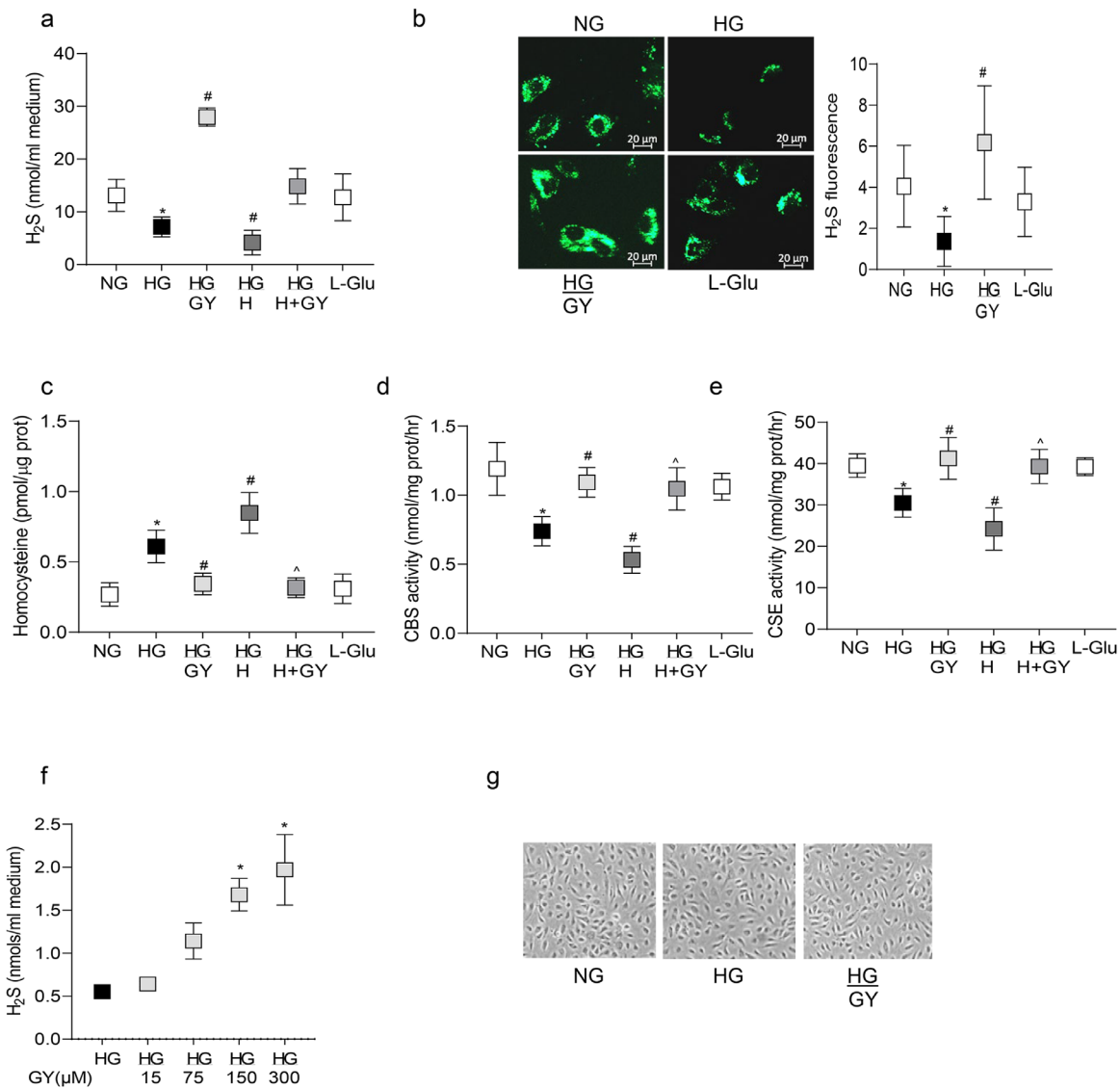


FIGURE 5. Hydrogen sulfide donor and glucose-induced alterations in H₂S and its enzymatic machinery in retinal endothelial cells. HRECs, incubated in high glucose, in the absence or presence of homocysteine, and without or with GY for 96 hours were analyzed for (a) extracellular H₂S level in their culture medium using NaHS as a standard, and (b) endogenous H₂S by in situ fluorescent microscopy using azido-4-methylcoumarin fluorescence probe. The accompanying histogram represents mean \pm SD from three different experiments, with each measurement performed in six or more cells. (c) Homocysteine levels were quantified using an ELISA kit, and (d, e) the activities of CBS and CSE were measured using spectrophotometric methods. Each measurement was made in duplicate or triplicate in four to five different cell preparations. (f) Concentration curve of GY on extracellular H₂S in the culture medium of HRECs incubated in high glucose. (g) HRECs incubated with 150 μ M GY visualized under an Olympus BX50 microscope and imaged at 10 times magnification. NG, cells in 5 mM glucose; HG and HG/GY, cells in 20 mM glucose without and with GY, respectively; HG/H and HG/H + GY, cells in high glucose + homocysteine, in the absence and presence of GY, respectively; L-Glu, cells in 20 mM L-glucose. * P < 0.05 versus NG; # P < 0.05 versus HG, and ^ P < 0.05 versus HG/H.

Diab group; Fig. 4a), and higher gene transcripts of *Cytb* and *ND6* (Figs. 4b, 4c).

Many patients with diabetes also have high homocysteine levels, and hyperglycemia and homocysteine can produce synergistic detrimental effects on the vasculature.³⁴ Effect of homocysteine supplementation on H₂S levels was determined in a hyperglycemic milieu. Homocysteine supplementation, soon after induction of diabetes in mice, despite producing no additional increase in retinal homocysteine (P > 0.05 versus Diab group), further decreased H₂S levels and reduced CBS and CSE activities; H₂S levels and enzyme activities were 20 to 30% lower in mice in the Diab/H group

compared to mice in the Diab group (P < 0.05 versus Diab; see Fig. 1). However, despite significant increase in oxidative stress and MMP-9 activity (P < 0.05 versus Diab group), supplemental homocysteine had no significant effect on the mitochondrial damage and retinal histopathology (P > 0.05 versus Diab group; Figs. 2–4); the values from diabetic mice with, or without, homocysteine was similar, but were significantly different from those obtained from normal mice (P < 0.05 versus Normal group).

Mice receiving simultaneous administration of homocysteine and GY had significantly higher retinal H₂S and CBS and CSE activities, compared to diabetic mice

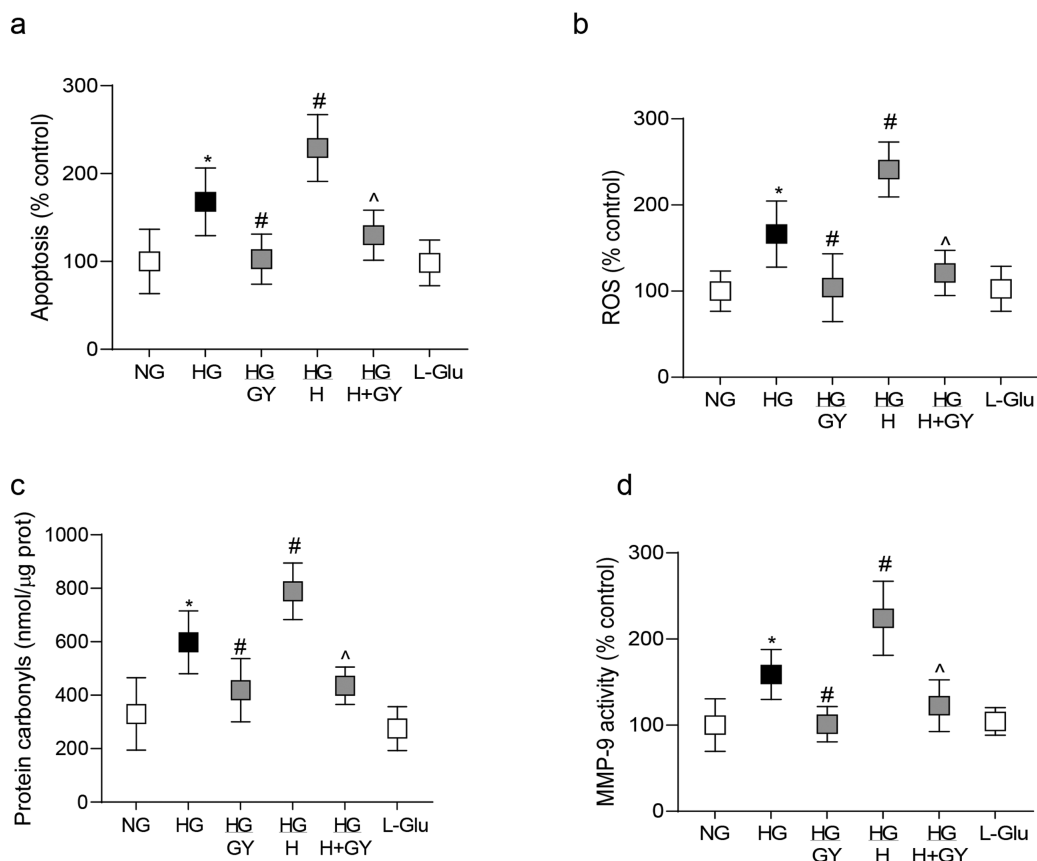


FIGURE 6. Hydrogen sulfide donor and glucose-induced cell apoptosis-oxidative stress and MMP-9 activation. HRECs were analyzed for (a) cell apoptosis using an ELISA method, (b) total ROS by DCFDA method, (c) protein carbonyls by employing 2,4-dinitrophenylhydrazine, and (d) MMP-9 activity using its fluorogenic substrate. Values obtained from cells in normal glucose are considered as 100%, and are represented as mean \pm SD from three different cell preparations, with each measurement performed in duplicate. * $P < 0.05$ versus NG; # $P < 0.05$ versus HG, and ^ $P < 0.05$ versus HG/H.

receiving homocysteine alone ($P < 0.05$ versus Diab + Homocysteine group; see Fig. 1), but GY administration failed to provide any beneficial effect on their retinal histopathology (acellular capillaries and pericyte ghosts) and oxidative stress-MMP-9-mitochondrial damage (Figs. 2–4).

Retinal Endothelial Cells

Compared to cells in normal glucose, high glucose decreased extracellular and intracellular H_2S by approximately 50% (Figs. 5a, 5b), increased homocysteine levels by twofold and inhibited CBS and CSE activities (Figs. 5c–e). Addition of 150 μM GY in high glucose medium significantly ameliorated decrease in H_2S , however, increasing GY concentration to 300 μM had no additional beneficial effect on H_2S levels; H_2S values from high glucose incubated cells with 150 μM GY or 300 μM GY were not significantly different from each other ($P > 0.05$). As a control, 15 μM GY failed to restore glucose-induced decrease in H_2S levels (Fig. 5f); subsequent experiments utilized 150 μM GY. Values obtained from cells incubated in 20 mM L-glucose, instead of 20 mM D-glucose, were significantly higher ($P < 0.05$), and were similar to those obtained from cells in normal glucose. HRECs incubated in high glucose, with or without 150 μM GY, had similar morphology as

seen in the cells incubated in normal glucose (Fig. 5g), suggesting that GY supplementation did not damage the cell phenotype.

Consistent with H_2S , GY supplementation also attenuated increase in cell apoptosis (Fig. 6a), oxidative stress (Figs. 6b, 6c) and MMP-9 activation (Fig. 6d), and prevented increase in mitochondrial ROS and membrane permeability (Figs. 7a, 7b) and mtDNA damage and transcription of *Cytb* (Figs. 7c, 7d). Compared to the HG group, while cell apoptosis, oxidative stress (protein carbonyls and ROS), MMP-9 activity, and mitochondrial damage (permeability and mtDNA damage) were significantly reduced in the HG/GY group, transcripts of *Cytb* were increased. Values from cells in 20 mM L-glucose were similar to those in normal glucose.

Compared to glucose alone, cells in high glucose medium, supplemented with homocysteine (HG/H group), had significantly lower H_2S and CBS and CSE activities ($P < 0.05$ versus HG; Fig. 5). Their cell death and oxidative stress-MMP-9-mitochondrial damage (membrane and DNA) were also worsened (Fig. 6, Fig. 7). Addition of GY in the high glucose medium containing homocysteine attenuated increase in homocysteine, and ameliorated decrease in H_2S and CBS-CSE activities (Fig. 5). Their cell apoptosis and oxidative stress MMP-9 activation-mitochondrial damage were also attenuated (Fig. 6, Fig. 7); the values from cells in the HG/GY and the HG/H + GY groups were

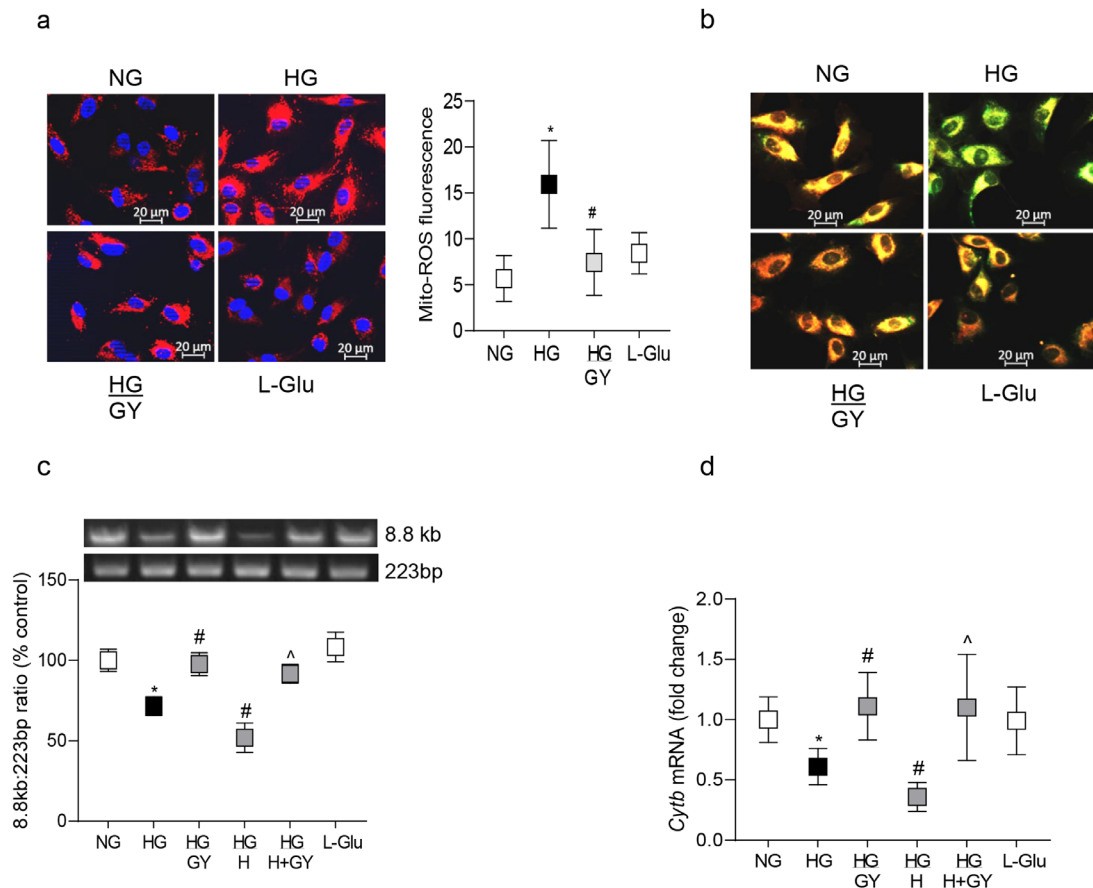


FIGURE 7. Glucose-induced mitochondrial damage and H₂S donor. **(a)** Mitochondrial ROS were quantified by MitoSOX Red and imaged at 20 times magnification using Apotome microscope, and the histogram represent the fluorescence intensity quantified by ImageJ, from 5 to 8 images/group. **(b)** Mitochondrial membrane potential was determined by cationic dye JC-1, and while *green fluorescence* represents the depolarized (monomer) mitochondria, *orange* represents hyperpolarized (J aggregates) mitochondria. **(c)** Damage to the mtDNA was quantified by extended-length PCR using long mtDNA (8.8 kb) and short (223 bp) amplicons of the mtDNA. **(d)** Gene transcripts of *Cytb* were quantified by qRT-PCR using β -actin as the housekeeping gene. Each measurement was made in duplicate/triplicate in four to five different cell preparations, and the histograms represent values mean \pm SD. NG, cells in 5 mM glucose; HG and HG/GY, cells in 20 mM glucose without and with GY, respectively; HG/H and HG/H + GY, cells in high glucose + homocysteine, in the absence and presence of, respectively; LGlu, cells in 20 mM L-glucose. * $P < 0.05$ versus NG; # $P < 0.05$ versus HG; and ^ $P < 0.05$ versus HG/H.

similar with each other, but were different from those obtained from cells without GY.

DISCUSSION

Hydrogen sulfide is recognized as a crucial gasotransmitter, which exerts many biological actions in various tissues including anti-inflammation and vasoregulation. It is synthesized endogenously from L-cysteine, and its generation is closely associated with homocysteine metabolism.^{35,36} Our previous work has shown that human donors with diabetic retinopathy have significantly reduced retinal H₂S levels and CBS activity, compared to their age-matched nondiabetic donors.¹⁶ H₂S displays significant antioxidant properties, and is now also considered as a signaling molecule.¹⁵ Here, using both in vitro and in vivo models of diabetic retinopathy, we have provided exciting results showing that a slow-releasing H₂S donor prevents the development of histopathology characteristic of diabetic retinopathy. We propose a possible mechanism that, by restoring H₂S levels, increase in oxidative stress-activation of MMP-9 is ameliorated. This protects mitochondrial integrity, and prevents

damage to the mtDNA and its transcription, protecting cells from undergoing accelerated apoptosis, and the development of diabetic retinopathy.

Normal plasma homocysteine levels range from 4 to 15 μ mol/L, but 5% to 12% of the general population has mildly elevated levels (hyperhomocysteinemia). High homocysteine is considered as a risk factor for several vascular diseases, including heart disease, and is also implicated in retinal disorders, including retinal vein occlusion, endothelial cell dysfunction,³⁷ and disruption of retinal pigment epithelium.^{38,39} Patients with diabetes generally present high plasma homocysteine levels, and the levels can go up to 50 to 100 μ mol/L,^{11,40} and clinical studies have shown a strong correlation between hyperhomocysteinemia and diabetic retinopathy.^{40,41} In physiological conditions, homocysteine and H₂S regulate each other,¹³ however, in patients with diabetes and animal models, plasma H₂S levels are significantly lower.^{42,43} Our previous study has shown greater than threefold elevation in retinal homocysteine in human diabetic donors with documented retinopathy compared to without retinopathy, and approximately twofold decrease in H₂S levels.¹⁶ Here, we show that retinal H₂S levels are

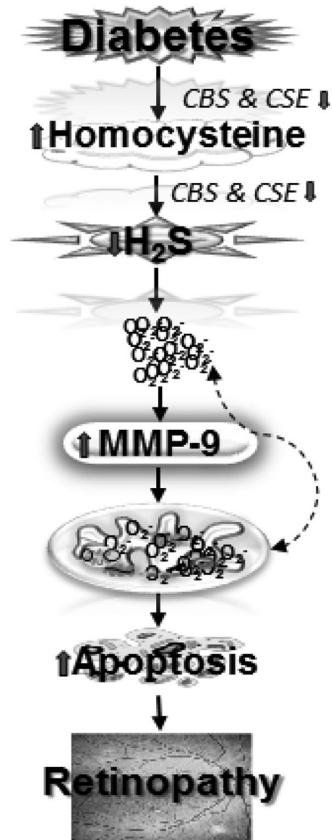


FIGURE 8. Schematic showing a possible mechanism by which decrease in H₂S in diabetes could result in diabetic retinopathy.

significantly lower in diabetic mice, and administration of a slow releasing H₂S donor, in addition to preventing decrease in H₂S, prevents the development of histopathology characteristic of diabetic retinopathy. In addition, GY also inhibits diabetes-induced retinal vascular leakage in these mice. In support, GY is shown to prevent retinal vascular leakage and abnormalities in retinal function in mice lacking *CBS*,⁴⁴ and its intravitreal administration preserves ganglion cells from acute ischemic injury and optic nerve crush, and by dilating retinal vessels, improves their perfusion.⁴⁵ In diabetes, GY protects accelerated atherosclerosis and inhibits inflammatory activation.⁴⁶ Supplementation with H₂S donor, sodium hydrosulfide (NaHS), in diabetic rat model improves retinal vascular permeability, neuronal dysfunction, and thickness,⁴⁷ and in the rat glaucoma model, provides neuroprotection and attenuates ganglion cell apoptosis.⁴⁸

Hydrogen sulfide is endogenously produced during homocysteine metabolism by CBS and CSE,^{13,49} and although both of these enzymes are expressed ubiquitously, CBS expression is mainly in the nervous system and CSE in the endothelial and vascular smooth muscle cells.⁵⁰ Our results clearly document that diabetes inhibits both of these enzymes in the retinal vasculature, and GY prevents their inhibition. Consistent with these, GY is shown to upregulate CBS in cardiomyocytes,¹³ and upregulates CSE in mice lacking *CBS*.⁴⁴

Hydrogen sulfide acts as an antioxidant; it can scavenge ROS directly, inhibit the biological activity of peroxynitrite, and enhance the levels of GSH.^{13,14} In diabetic retinopathy, oxidative stress mitochondrial damage plays a major

role.^{5,51} The data presented here show that GY inhibits diabetes-induced increase in oxidative stress and mitochondrial damage. These results are supported by others showing that NaHS protects elevation of oxidative stress and mitochondrial damage in an in vitro model of Parkinson's disease.⁵² In the pathogenesis of diabetic retinopathy, activation of MMP-9 damages the mitochondria, and MMP-9 itself is an oxido-sensitive enzyme.⁸ Here, we show that GY also ameliorates activation of MMP-9 in retinal microvasculature. In support, treatment of mice lacking *CBS* with NaHS ameliorates MMP-9 activation and microvascular permeability in the brain, in part, by normalizing the MMP/TIMP ratio.¹⁸ Addition of homocysteine in a diabetic milieu is shown to have synergistic detrimental vascular effects, and our recent study has shown that supplementation with homocysteine in hyperglycemic conditions exacerbates increase in ROS and MMP-9 activation.²³ Now, we show that hyperglycemia and homocysteine, by exacerbating inhibition of CBS and CSE, although further decrease H₂S levels and increase oxidative stress-MMP-9 activation, do not aggravate mitochondrial damage, and the severity of retinopathy in diabetic mice with, or without, homocysteine is similar. This could be that the decrease in H₂S, induced by supplemental homocysteine, might not be sufficient to further damage the mitochondria. Diabetic retinopathy is also considered as a low-grade inflammatory disease.⁵³ H₂S also affects inflammation, and GY is shown to inhibit inflammation-induced adipocyte dysfunction.⁵⁴ The possibility that GY could be ameliorating the development of retinal histopathology via inhibiting diabetes-induced retinal inflammatory mediators cannot be ruled out. In addition, we recognize that the results presented here are from both male and female mice; the influence of sex hormones on any of the parameters investigated in this manuscript cannot be ruled out.

Consistent with our in vivo results, supplementation of high glucose with GY in HRECs prevents increase in homocysteine, and decrease in H₂S and activities of CBS and CSE. Furthermore, GY also prevents increase in the oxidative stress-MMP-9 activation-mitochondrial damage, protecting endothelial cells from accelerated apoptosis, which, in animal models, is documented to precedes the development histopathology characteristic of diabetic retinopathy.² Addition of both homocysteine and GY in high glucose medium, normalizes H₂S levels, oxidative stress-MMP-9-mitochondrial damage, and prevents increase in cell apoptosis.

However, in contrast to our in vitro results, simultaneous supplementation of GY and homocysteine, soon after induction of diabetes in mice, despite increasing retinal H₂S levels, fails to provide any beneficial effect on retinal histopathology, and the numbers of acellular capillaries and pericyte ghosts continue to be high. One reason for the failure of GY to produce any effect on the retinopathy could be that the amount of H₂S failed to attain a threshold level to protect increase in oxidative stress-mitochondrial damage, and the damaged mitochondria continues to accelerate cell apoptosis. Our study has focused mainly on the retinal endothelial cells and the vasculature, similar detrimental effects of homocysteine on other retinal cell types that are also affected in diabetes, including Muller cells,⁵⁵ cannot be ruled out.

The preferable ocular drug delivery routes are the topical or systemic delivery, however, a well-organized blood-retinal barrier makes it challenging for the drugs to reach to the posterior segment of the eye. Intravitreal administration is routinely preferred for retinal diseases, but this

invasive technique carries some unwanted hidden long-term drawbacks.⁵⁶ Although we did not perform any pharmacokinetics of GY in the eye, the results presented here clearly document that the systemic administration of GY (at 0.25 mg/Kg) has beneficial effect in preventing the development of histopathology characteristic of diabetic retinopathy, suggesting that this slow-releasing small molecule can cross the blood-retinal barrier, reaching to the posterior segment of the eye.

In summary, we have identified a novel regulatory mechanism involved in the pathogenesis of diabetic retinopathy, specifically at the level of regulation of H₂S. The diabetic environment, by inhibiting CBS and CSE activities, increases homocysteine and reduces H₂S. This contributes to increased ROS accumulation, ROS, via activating MMP-9, damage the mitochondria, and the damaged mitochondria, in turn, continues to produce ROS. Retinal capillary cell apoptosis is accelerated, ultimately leading to the development of retinopathy (Fig. 8). H₂S donors ameliorate reduction in H₂S levels and protect MMP-9-mitochondrial damage, and this prevents/retards the development of diabetic retinopathy. Our results raise a possibility of the use of H₂S releasing/stimulating compounds for patients with diabetes to protect retinal damage, and prevent them from losing their vision from this debilitating disease. With recent technical advances, the field of H₂S releasing compounds has made great progress,^{49,57} and the future for developing controllable H₂S donors with different H₂S releasing mechanisms looks optimistic for patients with diabetes.

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Data availability: RAK is the guarantor of this work and, as such, has full access to all the data in the study. RAK takes responsibility for the integrity of the data and the accuracy of the data analyses.

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