



Hydrogen Sulfide Is a Novel Protector of the Retinal Glycocalyx and Endothelial Permeability Barrier

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Significantly reduced levels of the anti-inflammatory gaseous transmitter hydrogen sulfide (H₂S) are observed in diabetic patients and correlate with microvascular dysfunction. H₂S may protect the microvasculature by preventing loss of the endothelial glycocalyx. We tested the hypothesis that H₂S could prevent or treat retinal microvascular endothelial dysfunction in diabetes. Bovine retinal endothelial cells (BRECs) were exposed to normal (NG, 5.5 mmol/L) or high glucose (HG, 25 mmol/L) ± the slow-release H₂S donor NaGYY4137 *in vitro*. Glycocalyx coverage (stained with WGA-FITC) and calcein-labeled monocyte adherence were measured. *In vivo*, fundus fluorescein angiography (FFA) was performed in normal and streptozotocin-induced (STZ) diabetic rats. Animals received intraocular injection of NaGYY4137 (1 μM) or the mitochondrial-targeted H₂S donor AP39 (100 nM) simultaneously with STZ (prevention) or on day 6 after STZ (treatment), and the ratio of interstitial to vascular fluorescence was used to estimate apparent permeability. NaGYY4137 prevented HG-induced loss of BREC glycocalyx, increased monocyte binding to BRECs ($p \leq 0.001$), and increased overall glycocalyx coverage ($p \leq 0.001$). In rats, the STZ-induced increase in apparent retinal vascular permeability ($p \leq 0.01$) was significantly prevented by pre-treatment with NaGYY4137 and AP39 ($p < 0.05$) and stabilized by their post-STZ administration. NaGYY4137 also reduced the number of acellular capillaries (collagen IV + /IB4-) in the diabetic retina in both groups ($p \leq 0.05$). We conclude that NaGYY4137 and AP39 protected the retinal glycocalyx and endothelial permeability barrier from diabetes-associated loss of integrity and reduced the progression of diabetic retinopathy (DR). Hydrogen sulfide donors that target the glycocalyx may therefore be a therapeutic candidate for DR.

Keywords: glycocalyx, retinal permeability, diabetes, hydrogen sulfide, inflammation, mitochondria, slow-release hydrogen sulfide donors

INTRODUCTION

Loss of integrity of the vascular permeability barrier is associated with a range of pathological conditions. In particular, increased permeability is observed in diabetes, and in hyperglycemic conditions (Gillies et al., 1997; Brownlee, 2001; Allen and Bayraktutan, 2009; Saker et al., 2014) where it is associated with the pathogenesis of life-altering diabetic microvascular complications such as retinopathy (Sander et al., 2007). The vascular permeability barrier is formed of endothelial cells (ECs), their underlying basement membrane, and the endothelial glycocalyx. The latter is now recognized as a key regulator of permeability as demonstrated by a range of studies showing that selective loss of specific glycocalyx components is associated with altered permeability properties in various vascular beds (Jeansson and Haraldsson, 2003; Singh et al., 2007; Landsverk et al., 2012; Betteridge et al., 2017; Onions et al., 2019).

A role for endothelial glycocalyx damage in diabetes-associated vascular changes has been described. Glycocalyx reduction is associated with increased vascular permeability and increased leucocyte and platelet adhesion in acute hyperglycemia and diabetes (Nieuwdorp et al., 2006a,b; Broekhuizen et al., 2010; Kumase et al., 2010), which were reversed by glycocalyx restoration experimentally in animals (Henry and Duling, 1999; Constantinescu et al., 2003). Strategies to reverse or reduce glycocalyx damage in diabetes have the potential to rescue some of the potentially damaging vascular changes that underlie the development of vascular complications such as retinopathy.

We hypothesized that exogenous application of hydrogen sulfide (H_2S) may protect the glycocalyx in diabetes, reversing or preventing the retinal vascular leakage observed in diabetic retinopathy (DR). Hydrogen sulfide has recently been identified as a seemingly ubiquitous “gaseous mediator” in mammals and humans where it is synthesized by multiple cell types by at least four distinct enzyme systems utilizing endogenous sulfur-containing amino acids. These systems include the cytosolic cystathionase (CSE), cystathionine- β -synthase (CBS), mitochondrial cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (CAT/3MST), and D-amino acid oxidase/3-mercaptopyruvate sulfurtransferase (DAO/3MST) (Whiteman et al., 2011; Szabo, 2012; Wang, 2012; Szabo et al., 2014). The first three, at least have been demonstrated in ECs (Wang, 2012; Tao et al., 2013; Mitidieri et al., 2020). Experimentally, manipulation of H_2S level has been achieved by pharmacological inhibition or genetic removal of enzyme systems for H_2S and the use of H_2S delivery molecules, albeit generally limited to the use of simple inorganic sulfide salts [e.g., sodium hydrosulfide (NaSH) or disodium sulfide (Na_2S)]. These studies have revealed that H_2S and/or physiologically derived species exert a wide variety of effects on different organ systems including regulation of vascular tone (Yang et al., 2008; Kanagy et al., 2017; Szabo, 2017), inflammation (Whiteman and Winyard, 2011), aging and health span (Ng et al., 2018; Zivanovic et al., 2019), and more recently as a regulator of retinal physiology (Du et al., 2017).

There is increasing evidence for impaired vascular EC synthesis and/or bioavailability of H_2S in diabetes (Szabo, 2012). Lower vascular and/or tissue levels of “ H_2S ” have been observed

in humans with diabetes (Whiteman et al., 2010a; Suzuki et al., 2017), and in several animal diabetic models, including streptozotocin (STZ)-treated rats (Yusuf et al., 2005; Si et al., 2013; Zhou et al., 2014) and mice (Suzuki et al., 2011) and in db/db (Peake et al., 2013; Sun et al., 2018), ob/ob (Zhao et al., 2017), Akita (Kundu et al., 2013; John et al., 2017), and NOD (Brancaleone et al., 2008) mice. Administration of often high doses of H_2S via sulfide salts at least partially prevented the diabetic phenotype in some of these studies. In the STZ diabetes model specifically, retinal levels of H_2S were lower in diabetic animals compared with controls (Si et al., 2013), and retinal capillary leakage, VEGF levels, and oxidative stress markers were partially normalized after NaSH treatment (Si et al., 2013). NaSH administration also partially restored mitochondrial function, e.g., increased ATP synthesis and complex II and III activity and prevented mitochondrial oxidant production and mitochondrial swelling, demonstrating a likely mitochondrial target for pharmacological H_2S (Si et al., 2013). Collectively, these studies strongly suggest that diabetes and diabetic retinopathy (DR) result from “ H_2S deficiency,” which could at least be partially overcome by pharmacological manipulation of mitochondrial H_2S levels.

It is important to note that these previous studies on H_2S and diabetes have exclusively relied on the use of sulfide salts to generate H_2S (e.g., NaSH and Na_2S). While useful laboratory tools for H_2S generation, they are not without severe limitations (Whiteman et al., 2011). These include generation of an instant, and unphysiological bolus of H_2S by pH-dependent dissociation (and rapid decay), whereas endogenous organic enzymatic H_2S synthesis produces low levels of H_2S over prolonged periods of time (Li et al., 2008; Whiteman et al., 2010b, 2011; Kabil and Banerjee, 2014).

It is now recognized that mitochondrial oxidative stress may contribute to the pathogenesis of diabetic endothelial dysfunction (Brownlee, 2005) and that in diabetes, the primary target for H_2S activity appears to be the mitochondria where it is used as an electron source for respiration to reduce oxidative stress (Módis et al., 2014; Szabo et al., 2014). This is supported by data indicating that mitochondrial-targeted slow-release H_2S donors preserved cellular bioenergetics and increased levels of Nrf2 and peroxisome proliferator-activated receptor- γ coactivator (PGC-1 α), key regulators of mitochondrial antioxidant capacity, bioenergetics, and biogenesis, both *in vitro* and *in vivo* in response to UVA-induced photoaging (Lohakul et al., 2021).

However, H_2S concentrations (derived from sulfide salts) that are required to reverse mitochondrial dysfunction in the vasculature (e.g., in diabetic rats) are unattainable (Suzuki et al., 2011; Montoya and Pluth, 2016). This is presumably because H_2S from these tools is generated immediately, locally, and not targeted to mitochondria. With the development of the slow-release cytosolic donor sodium GYY4137 (Li et al., 2008) and the mitochondria-specific sulfide donor AP39 (Le Trionnaire et al., 2014) the limitations of NaSH/ Na_2S have been overcome. The effects on microvascular EC and in the kidney have been previously reported (Ahmad et al., 2016; Geró et al., 2016), but to the best of our knowledge, no previous studies have reported on the effects of these molecules in DR.

In this current study, we investigated the effects of a single dose (administered by intravitreal injection) of two slow-release H₂S donor molecules, the cytosolic sodium GYY4137 (Li et al., 2008) and the mitochondria-specific sulfide donor AP39 (Le Trionnaire et al., 2014), on retinal vascular permeability *in vivo* in the Norway Brown rat streptozotocin-induced DR model. In addition, the effects of these two H₂S generators on *in vitro* EC glycocalyx thickness using bovine retinal endothelial cells (BRECs) were studied. Leucocyte adhesion to BRECs and the effects of the H₂S modulation were also evaluated in normal and high-glucose conditions.

MATERIALS AND METHODS

H₂S Donor Synthesis

The mitochondria-targeted H₂S donor AP39 was synthesized in our laboratories as previously described by our team (Le Trionnaire et al., 2014). Commercially available GYY4137 is a morpholine salt that contains unstated quantities of dichloromethane residual from its initial synthesis and which forms part of the lattice structure (at least 2 dichloromethane:1 GYY4137 molecule) (Alexander et al., 2015), i.e., it is commercially available as xCHCl₂ or a dichloromethane complex. Dichloromethane is metabolized *in vivo* to carbon monoxide (Ratney et al., 1974; Takano and Miyazaki, 1988). Furthermore, the morpholinium counter ion (1 morpholine:1 GYY4137 molecule) is not biologically inert and has a half-life in rats of ~90 min (Sohn et al., 1982). LD₅₀ values for morpholine in rats and mice, e.g., 100–400 mg/kg for i.p. administration (Kielhorn and Rosner, 1996), are well within the commonly used doses of commercial GYY4137, e.g., 50–300 mg/kg (Lee et al., 2011; Li et al., 2013). In the eye, morpholine and dichloromethane have been reported to cause keratoconjunctivitis, focal/diffuse cataract formation, keratitis, iritis, conjunctival epithelial edema and detachment, and denudation of corneal epithelium (Ballantyne et al., 1976; Brandt and Okamoto, 1988; Toxicology–cosmetic ingredient review, 1989). To avoid these clinical indications and associated toxicity, a cleaner dichloromethane and morpholine-free sodium salt synthesized as previously described was used in this study (Alexander et al., 2015).

Cell Culture and Treatments

Primary cultures of BRECs were isolated from bovine retinae dissected from eyes of freshly slaughtered cattle, by homogenization and a series of filtration steps as previously described (Chibber et al., 2000). Briefly, excess fat and muscle tissue was removed from the eye globes and the retina were dissected, placed in 20 ml of minimal essential medium (MEM) and homogenized using a hand-held sterile glass homogenizer to dissociate the neural retina. The resulting homogenate was filtered through 80- μ m nylon mesh, the material remaining on the mesh was collected, and the trapped microvessels were resuspended in serum free MEM with collagenase-dispase (2 mg/ml) and digested for 90 min at 37°C on a rotator shaker. Following further filtration through a 45- μ m polypropylene net filter, the trapped microvessel fragments were vigorously

pipetted in MEM with 10% (v/v) pooled human serum, 10% (v/v) tryptose phosphate broth (TPB), L-glutamine 2 mM, penicillin (100 U/ml), and streptomycin (100 μ g/ml) and plated in fibronectin-coated tissue culture flasks. After 24 h, the flasks were rinsed once with MEM to remove debris and unattached cells and refilled with fresh growth medium [MEM supplemented with 10% (v/v) pooled human serum, 10% (v/v) tryptose phosphate broth (TPB), L-glutamine 2 mmol/L, and penicillin (100 U/ml) and streptomycin (100 μ g/ml)]. From passage 1 onward, the BRECs were cultured on gelatin-coated tissue culture flasks and human serum was replaced with 10% (v/v) horse serum. Pericyte contamination was removed by differential trypsinization. Cultures were >90% pure as assessed by morphology and staining for von Willebrand factor. Preliminary studies confirmed that treatment with 25 mmol/L D-mannitol as osmotic control had no effect on either the glycocalyx or leukocyte adhesion compared with the normal glucose (NG) treatment. U937 cells (human leukemic monocyte lymphoma cell line) were cultured in RPMI-1640 with 10% (v/v) fetal bovine serum, 5.6 mmol/L D-glucose, 25 mmol/L HEPES, and antibiotics. Based on our preliminary results, a final concentration of 500 μ mol/L of NaGYY4137 was used in the definitive experiment. NaGYY4137 (500 μ mol/L) generates approximately 1 μ mol/L or less of H₂S during the incubation period (Szabo, 2017).

Glycocalyx Assessment

A cell-based fluorescent assay, based on that described by Singh et al. (2007), was performed to quantify changes within the glycocalyx using fluorescein isothiocyanate-labeled wheat germ agglutinin (WGA-FITC). Bovine retinal endothelial cells were exposed to normal (NG, 5.5 mmol/L) or high glucose (HG, 25 mmol/L) \pm GYY4137 (500 μ mol/L) in MEM with 0.5% bovine serum albumin (BSA, w/v) for 24 h. Cells were washed 3 \times with phosphate buffered saline (PBS) and incubated with WGA-FITC [2 μ g/ml in HEPES-buffered phenol red-free MEM containing 0.5% BSA (w/v)] for 30 min at 37°C. After washing with PBS (3 \times), fluorescence intensities were measured (Ex/Em, 485/520 nm) using a fluorescence microplate reader. Cells were lysed and the protein content of each well was measured using the bicinchoninic acid method. Results are presented as fluorescence unit/ μ g of protein.

Leukocyte Adhesion Assay

Bovine retinal endothelial cells (BRECs) were cultured to confluency in commercially available μ -slide VI.0.4 perfusion slides (Ibidi, München, Germany) coated first with 2% (w/v) bovine gelatin (2 h) and then overnight with bovine fibronectin (50 μ g/ml). Cells were treated with NG or HG as above. Calcein acetoxymethyl ester labeled (0.5 μ mol/L for 30 min) U937 cells (1 \times 10⁶/ml) were then flowed over confluent BREC cultures at a laminar shear stress of 1 dyne/cm² generated by a peristaltic pump. This shear stress was chosen to mimic venular wall shear stresses that favor leukocyte adhesion *in vivo* (Jones et al., 1995; Sundd et al., 2011). After 6 min, slides were washed with PBS for 1 min to remove non-adherent cells, fixed in 4% (w/v) paraformaldehyde, and examined using a Nikon Eclipse

TS100 (Nikon UK Limited, Kingston upon Thames, Surrey, United Kingdom) fluorescence microscope to assess the number of adherent U937 cells (10 random fields of view/slide). All experiments were performed on at least three separate occasions. Statistical significance was tested using the Student *t*-test.

Animal Ethics

Experimental animals were treated in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under a UK Home Office license at the University of Nottingham Biological Services Unit.

Streptozotocin-Induced Diabetes

A total of 12 male Norway Brown rats (250–300 g, Envigo, United States) were weighed and given a single intraperitoneal (i.p.) injection of STZ (50 mg/kg, Sigma–Aldrich, MO, United States). A total of six control rats were injected with 300 μ l of saline i.p. On days 0 and 4 and prior to sacrifice (day 7), blood glucose levels were tested using a sample of blood taken from the tail vein and an Accucheck blood glucose monitor. Rats with blood glucose levels of 15 mmol/L and above were deemed diabetic. Streptozotocin-injected rats that did not become hyperglycemic on day 4 were re-injected with STZ the following morning and subsequently included if deemed diabetic following evaluation for diabetes as outlined above.

Intravitreal Injections

Rats were anaesthetized with a single 10 mg/ml i.p. injection of Domitor (medetomidine hydrochloride, Pfizer, United Kingdom) and Ketaset (ketamine hydrochloride, Zoetis, NJ, United States). Pupils were dilated with topical applications of 5% (w/v) phenylephrine hydrochloride (Bausch and Lomb) and 0.8% (w/v) tropicamide (Bausch and Lomb), and eyes were coated with Lubrithal (*Dechra*) to prevent dehydration. A 1.5-cm 34-gauge hypodermic needle (Hamilton, NV, United States) attached to a 5- μ l syringe (World Precision Instruments, FL, United States) was inserted through pars plana at 3 mm from the limbus into the vitreous of the left eye at a 45° angle. Rats received 5 μ l of sterile PBS, 1 μ mol/L NaGYY4137 [sodium 4-methoxyphenyl(morpholino)-phosphinodithioate], or 100 nmol/L AP39 [(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide] on day 0 (prevention arm) or day 6 (treatment arm).

Fundus Fluorescein Angiography

Angiography was performed as described (Allen et al., 2020). Sodium fluorescein (NaF) dye was prepared by dissolving in sterile PBS to give a final concentration of 100 mg/ml and sterile filtered (0.2 μ m). Rats were anesthetized as previously described and fundus images of the retina were captured to check for any ocular abnormalities. Rats received a single 250 μ l i.p. injection of sodium fluorescein (100 mg/ml), which was allowed to circulate for ~60 s before imaging with a Micron IV Retinal Imaging Microscope (Phoenix Research Labs). The green filter was selected, and a 3-min video footage of the retina was recorded at 15 frames per second. This was carried out on days 0 and

7. Development of cataracts with resultant blurring of posterior segment view meant that some eyes ($n = 2$) were excluded from the consecutive fundus fluorescein angiography (FFA).

Retinal Permeability Model

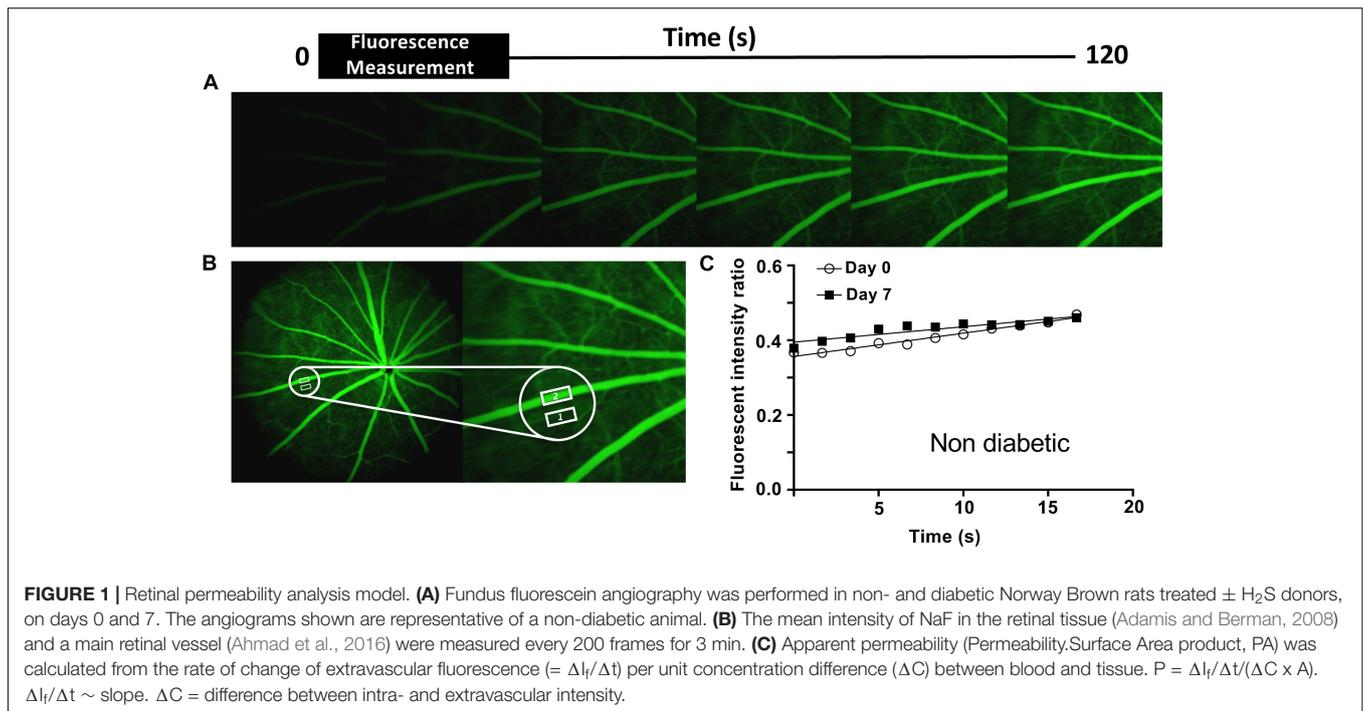
Angiograms were imported into ImageJ software and fluorescence was measured in the interstitium and a major retinal vessel every 200 frames up to 2,400 frames. The ratio of interstitial to vascular fluorescence was adjusted for background level and plotted against time and the slope used to determine an estimate of permeability (Figure 1). Figure 1A shows images taken from rats given NaF and recording started within 1 min of injection. The large vessels of the retina are clearly seen, and the retina becomes brighter over time. To estimate a measure of permeability, the fluorescence intensity in an area with no large vessels was measured. The solute flux (number of fluorescence molecules entering the interstitium per unit time) was calculated from the change in fluorescence intensity in the window outside the major vessel, as a proportion of the fluorescence intensity in the blood vessel in the same frame, to account for any changes in focus, excitation intensity, or noise, plotted against the time in seconds. The permeability surface area product was calculated from the solute flux, the concentration gradient between the intravascular and extravascular compartments (difference in fluorescence intensity), and the area of the extravascular sample measurement according to Fick's law. In healthy Norway Brown rats, the permeability was the same 7 days after intraocular injection with 2 μ l saline ($7.47 \pm 1.74 \times 10^{-4} \text{ s}^{-1}$) as it was before injection ($6.57 \pm 2.13 \times 10^{-4} \text{ s}^{-1}$). Streptozotocin treatment resulted in a significant increase in blood glucose ($29.73 \pm 0.66 \text{ mmol/L}$) after 1 week (compared with $7.92 \pm 0.37 \text{ mmol/L}$), which was accompanied by a significant increase in solute flux in the retina (Figure 4), which translated to an increase in estimated permeability from $8.19 \pm 0.95 \times 10^{-4} \text{ s}^{-1}$ before STZ to $13.32 \pm 1.65 \times 10^{-4} \text{ s}^{-1}$ after STZ induction (Allen et al., 2020).

Immunofluorescence

After animal termination and ocular dissection (day 14), retinæ were flat-mounted and blocked in 5% (v/v) goat serum, 3% (v/v) Triton X-100, 1% (w/v) BSA, and stained with isolectin-B4 (IB4) (Sigma Aldrich, biotin conjugated) 5 μ g/ml and rabbit anti-collagen IV (Abcam) 5 μ g/ml overnight at 4°C. Streptavidin conjugated Alexafluor 488 2 μ g/ml and donkey anti-rabbit Alexafluor 555 4 μ g/ml were used to detect IB4 and collagen IV staining, respectively. Coverslips were mounted with Fluoroshield with DAPI. Images were obtained using a Leica TCS SPE confocal microscope, and all settings were maintained between images.

Statistics

All statistics and graphs were produced in GraphPad Prism 6 and statistical tests are shown in figure legends. Significant differences are indicated on graphs as asterisks, where: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.



RESULTS

Glycocalyx Integrity

Two hydrogen sulfide generating molecules were investigated, NaGYY4137 and AP39. The former is a slow-releasing hydrogen sulfide donor (Alexander et al., 2015) that does not target any specific part of the cell, whereas AP39 is a mitochondrial targeted donor (Le Trionnaire et al., 2014).

HG significantly reduced BREC glycocalyx *in vitro* (assessed by FITC-WGA staining) to $89.67\% \pm 6.6\%$ compared with NG control conditions (100%), $p \leq 0.001$, $n = 12-30$ (Figure 2). Simultaneous treatment with the slow-release H₂S donor NaGYY4137 completely reversed this loss and actually restored the glycocalyx to higher levels than observed in the NG control ($107.49 \pm 8.13\%$ vs. 100%, $p \leq 0.001$, $n = 12-30$). Interestingly, cells incubated in NG conditions in the presence of NaGYY4137 also had significantly higher glycocalyx staining than control NG-treated cells (106.7 ± 6.35 vs. 100%, $p \leq 0.001$, $n = 12-30$).

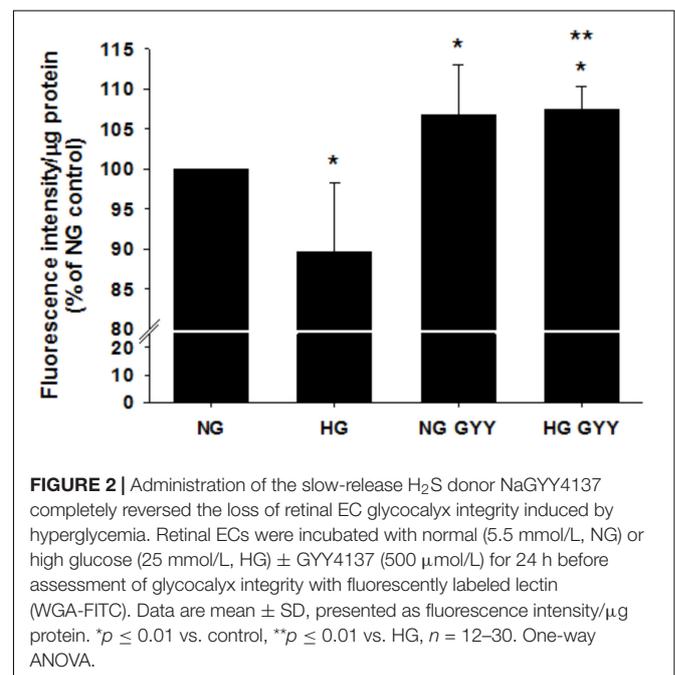
Since the integrity of the glycocalyx may alter endothelial cell adhesion molecule exposure and thus influence leukocyte/endothelial binding, adhesion of U937 leukocytes to a BREC monolayer was examined under the same experimental conditions. HG significantly increased leukocyte adhesion to $348 \pm 110\%$ vs. NG control (100%), $p \leq 0.01$, $n = 4$ (Figure 3). This increase was fully attenuated to control levels by incubation with NaGYY4137 (HG + NaGYY4137; $124 \pm 47\%$ vs. HG; $348 \pm 110\%$, $p \leq 0.01$, $n = 4$). NaGYY4137 had no significant effect on leukocyte binding in NG conditions.

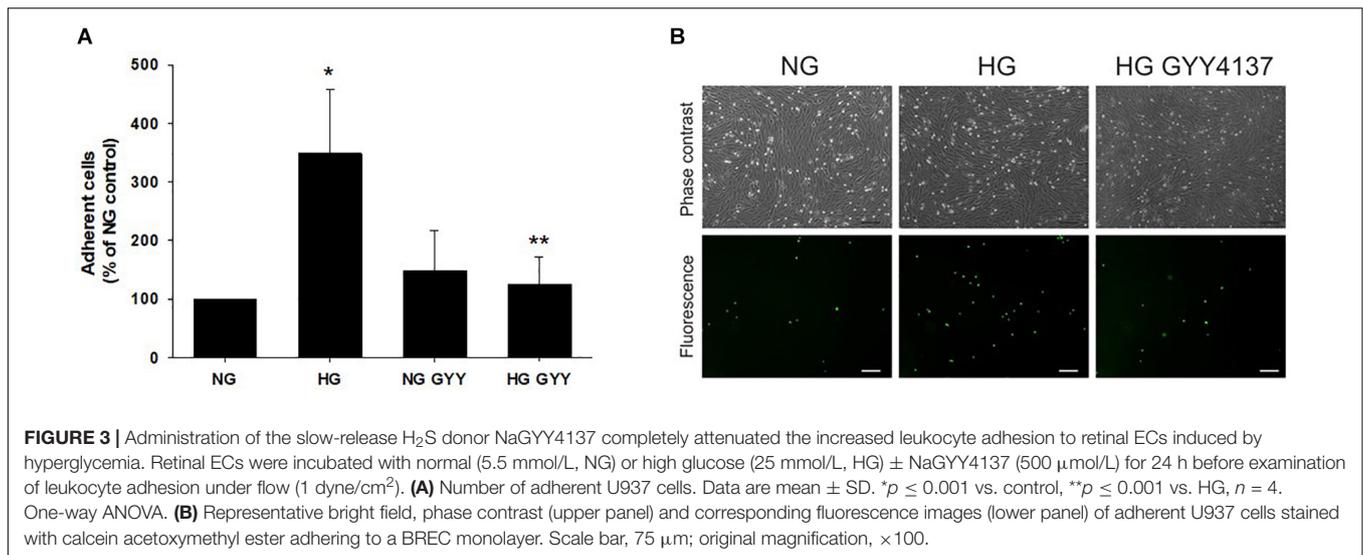
Retinal Permeability

To determine the effect of hydrogen sulfide donors on retinal permeability, fluorescein fundus angiography was carried out

before and after induction of diabetes with STZ. NaF was injected intraperitoneally into Norway Brown rats and the fluorescence intensity inside a large vessel and in the tissue outside a large vessel was determined over time by video-microscopy.

Retinal vascular permeability was significantly increased in the diabetic Norway Brown rats ($23.51 \pm 4.59 \times 10^{-4} \text{ s}^{-1}$, $n = 3$) compared with normal control rats ($9.38 \pm 1.40 \times 10^{-4} \text{ s}^{-1}$, $n = 6$, Figure 4) on day 28 ($p \leq 0.001$) on day 28 and all other time points. In the H₂S donor treatment study no changes





in retinal permeability were measured on day 7 compared to baseline in non-diabetic animals treated with vehicle control (saline) (**Figure 5A**). However, a significant ($p \leq 0.05$) increase in retinal permeability was measured in non-insulin treated diabetic animals on day 7 ($12.16 \pm 0.98 \times 10^{-4} \text{ s}^{-1}$) compared to baseline ($9.23 \pm 1.54 \times 10^{-4} \text{ s}^{-1}$) (**Figures 5B,C**). The slow-release hydrogen sulfide donor NaGY4137 significantly reduced retinal vascular permeability in control, non-diabetic rats after 7 days ($3.24 \pm 0.66 \times 10^{-4} \text{ s}^{-1}$, $p \leq 0.05$, $n = 6$) vs. day 0 ($10.12 \pm 1.85 \times 10^{-4} \text{ s}^{-1}$) when administered as a single preventative intraocular dose pre-diabetes (**Figure 5D**). Similarly, NaGY4137 given in diabetic rats significantly reduced retinal permeability on day 7 ($6.62 \pm 1.16 \times 10^{-4} \text{ s}^{-1}$, $p \leq 0.05$, $n = 6$) vs. day 0 ($13.08 \pm 2.61 \times 10^{-4} \text{ s}^{-1}$) when given as a preventative treatment (**Figure 5D**). When NaGY4137 was administered therapeutically, retinal permeability was reduced in control, non-diabetic animals on day 7 ($5.31 \pm 1.24 \times 10^{-4} \text{ s}^{-1}$, $n = 6$) vs. day 0 ($8.90 \pm 1.19 \times 10^{-4} \text{ s}^{-1}$, $n = 6$). More importantly, in animals with pre-existing diabetes, retinal permeability stabilized on day 7 ($10.27 \pm 1.21 \times 10^{-4} \text{ s}^{-1}$, $n = 6$) compared to day 0 ($8.65 \pm 1.23 \times 10^{-4} \text{ s}^{-1}$, $n = 6$) (**Figure 5E**). Administering NaGY4137 treatment prior to the onset of diabetes gave a 68% benefit in reducing retinal permeability compared with administration to animals with pre-existing diabetes (**Figure 5F**).

Similarly, the mitochondrial-targeted hydrogen sulfide donor AP39 significantly reduced retinal permeability in control, non-diabetic ($6.14 \pm 0.90 \times 10^{-4} \text{ s}^{-1}$, $p \leq 0.05$, $n = 6$) and diabetic rats ($9.84 \pm 1.48 \times 10^{-4} \text{ s}^{-1}$, $p \leq 0.05$, $n = 6$) compared with day 0 ($10.01 \pm 1.34 \times 10^{-4} \text{ s}^{-1}$ and $15.52 \pm 2.75 \times 10^{-4} \text{ s}^{-1}$, respectively) when administered as a single preventative dose (**Figure 5G**). In addition, AP39 reduced retinal permeability after a week ($5.93 \pm 2.25 \times 10^{-4} \text{ s}^{-1}$, $n = 6$) compared with baseline ($9.49 \pm 1.28 \times 10^{-4} \text{ s}^{-1}$, $p \leq 0.05$, $n = 6$) in control animals when administered therapeutically. However, in diabetic animals, AP39, when administered therapeutically, stabilized retinal permeability ($13.63 \pm 2.56 \times 10^{-4} \text{ s}^{-1}$, $n = 5$) on day 7 versus baseline ($8.99 \pm 1.31 \times 10^{-4} \text{ s}^{-1}$, $n = 5$) (**Figure 5H**). Similar to NaGY4137, administering AP39 treatment prior to

the onset of diabetes gave an 18% benefit in reducing retinal permeability when compared with animals with pre-existing diabetes (**Figure 5I**). Overall, both H₂S donors were more effective in reducing retinal permeability when given to animals prior to the onset of diabetes. In addition, NaGY4137 showed greater efficacy than a 10-fold lower dose of the mitochondrial targeted AP39 compound.

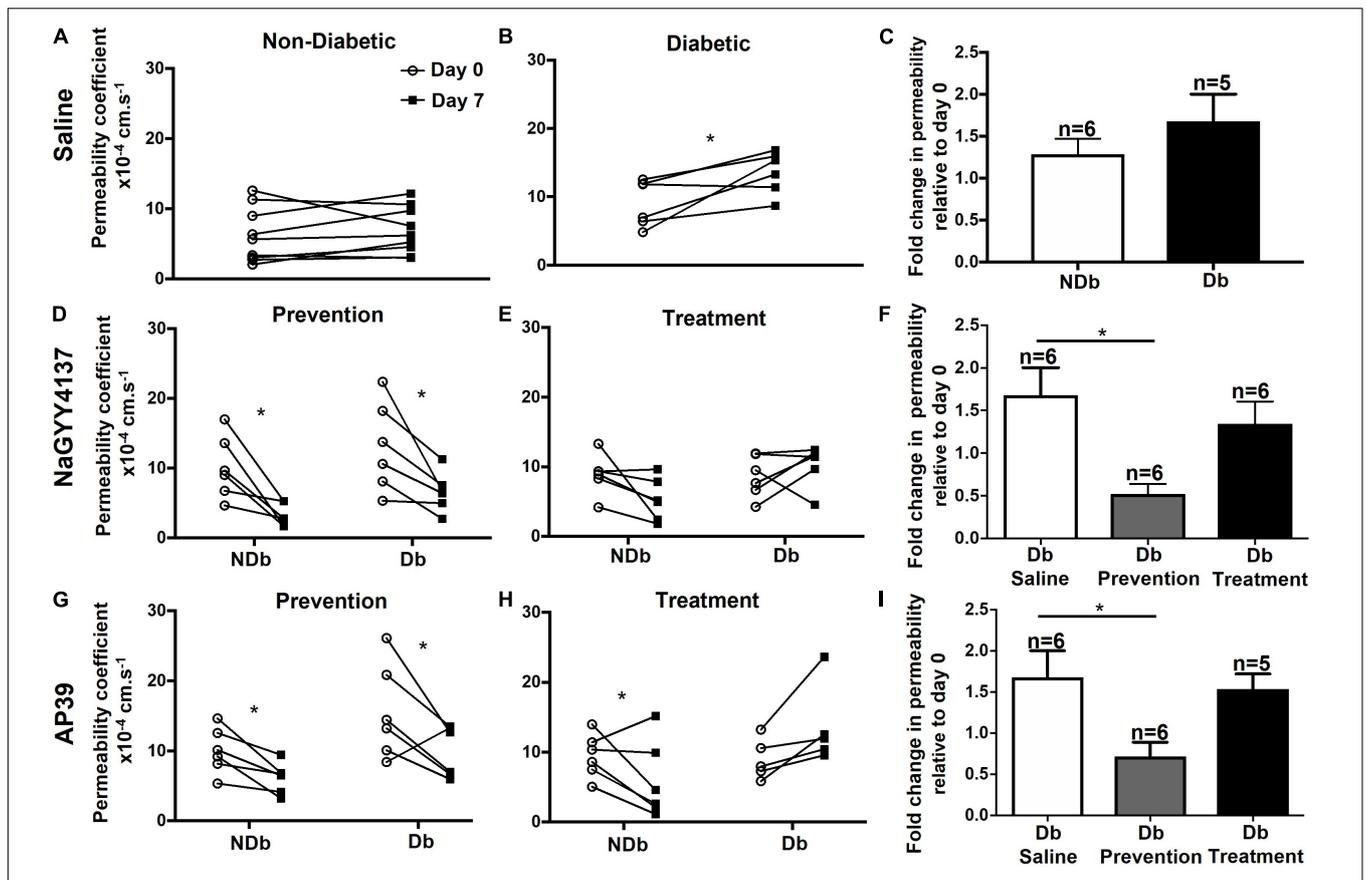
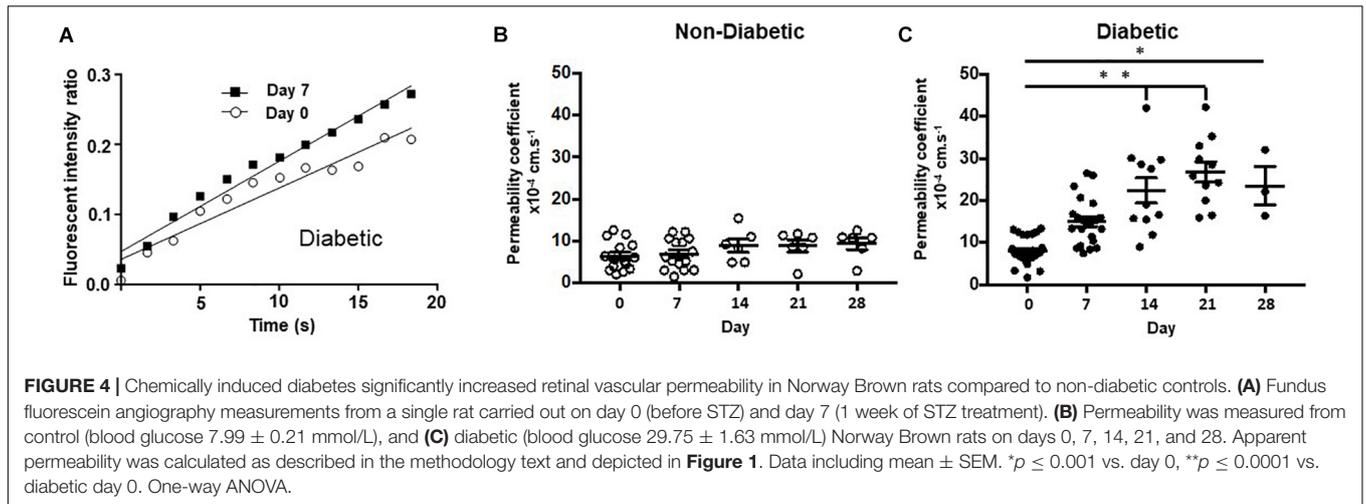
Acellular Capillary Formation

One of the contributing factors to the pathogenesis of DR is the presence of acellular capillaries within the retina. These vessels maintain a basement membrane but have lost the supporting pericytes and EC cells and thus have no blood flow, promoting retinal ischemia. In order to assess the effects of NaGY4137 on acellular capillary formation, retinae were collected at the end of the retinal permeability study and stained for isolectin B4, an EC marker, and collagen IV, a major constituent of the basement membrane. NaGY4137 was found to reduce the number of acellular capillaries (collagen IV + /IB4-) in the diabetic retinae in both the prevention and treatment group to $80.2 \pm 13.9 \text{ mm}^{-2}$ and $66.1 \pm 7.1 \text{ mm}^{-2}$, respectively, compared with $113.6 \pm 11.7 \text{ mm}^{-2}$ in the diabetic retinae ($p \leq 0.05$, $n = 3$, **Figure 6**). Despite the increased effectiveness of NaGY4137 in reducing retinal permeability when administered prior to diabetes onset, the time of administration appeared to have no difference in acellular capillary formation.

DISCUSSION

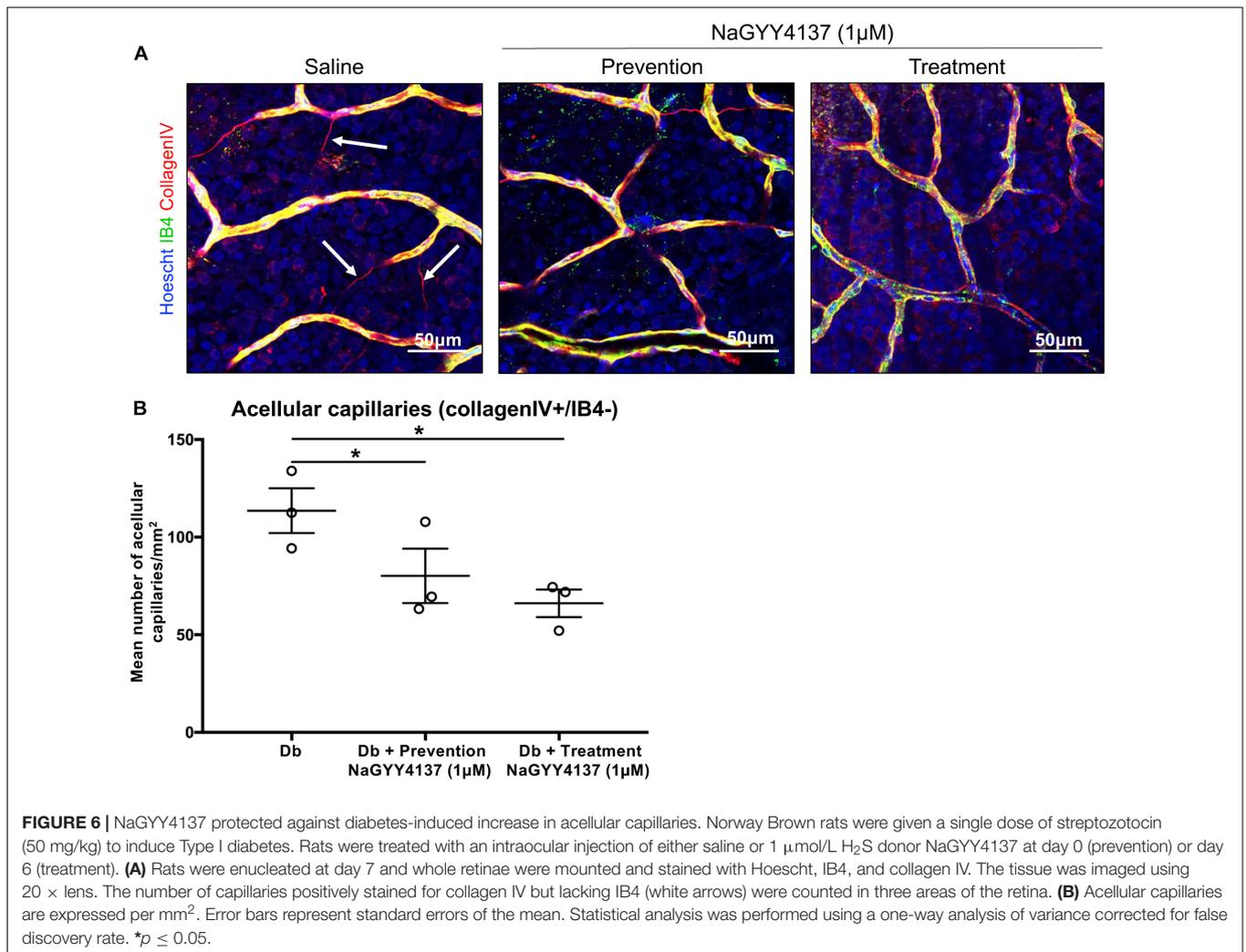
The data presented here indicate, for the first time, that (a) high glucose-driven glycocalyx loss, (b) high glucose-enhanced leukocyte adhesion to the endothelium, and (c) diabetes-associated increases in retinal permeability can all be reversed by administration of a slow-release H₂S donor.

Hyperglycemia-related low-grade persistent inflammation is thought to contribute to the pathology of early DR (Adams and Berman, 2008). Patients with DR have elevated ocular



levels of inflammatory mediators and the diabetic retina displays characteristic features of inflammation including increased vascular permeability and leukocyte adhesion (Adamis and

Berman, 2008). Since a healthy glycocalyx is critical to maintenance of both the anti-inflammatory and permeability characteristics of a healthy endothelium, our data highlight



the potential of H₂S donors as new therapeutic tools for the treatment of DR. Further investigations into optimal dosing and comparing other H₂S delivery molecules are needed to further explore this potential.

The available literature describing other research in this area is scarce. However, glycocalyx loss and enhanced leukocyte adhesion have been reported in a rat model of diabetes (Kumase et al., 2010). Additionally, a clear link between *in vivo* retinal glycocalyx integrity and vascular permeability has been reported by Leskova et al. (2019), who demonstrated, in mice, that enzymatic degradation of the glycocalyx is associated with enhanced retinal permeability. These data support the hypothesis that maintaining glycocalyx integrity could be a viable therapeutic strategy for DR. EC glycocalyx thickness depends on the rate of shedding and circulatory levels of glycosaminoglycans (GAG)-degrading enzyme levels (Ikegami-Kawai et al., 2003; Maxhimer et al., 2005). The previously reported association of glycocalyx reduction with increased vascular permeability and leukocyte and platelet adhesion and subsequent reversal by glycocalyx restoration in experimental animals through increased GAG synthesis (Henry and Duling, 1999; Constantinescu et al., 2003) corroborate

our findings. Furthermore, Ciszewicz et al. (2009), have shown that sulodexide attenuates hyperglycemia-associated EC permeability and inflammation. Similarly, Broekhuizen et al. (2010), demonstrated that sulodexide increased vascular EC glycocalyx and reversed the increased vascular permeability in type 2 diabetes. Additionally, Niu et al. (2019), using the STZ-induced diabetic rat model used here, showed that endomucin overexpression restored the diabetes-associated loss of retinal endothelial glycocalyx and that this restoration was associated with decreased leukocyte-endothelial adhesion and a reduction in vessel leakage in rats with DR.

The effects of H₂S on the glycocalyx are still unknown. Although early studies showed potential antioxidant effects of H₂S, more recent work has shown that the reaction rate between H₂S and oxidant species, e.g., peroxide and peroxyxynitrite, may be too slow for oxidant scavenging activity alone to be primarily responsible for glycocalyx- and vaso-protection, especially given the low *in vivo* concentrations of H₂S, which we have modeled using NaGYY4317 (Carballal et al., 2011). This strongly suggests that H₂S is acting *via* other mechanisms to maintain the glycocalyx during hyperglycemic damage, e.g., enhancing synthesis of glycocalyx components or preventing

hyperglycemia-induced glycocalyx degradation. For instance, the glycocalyx component syndecan-1 is cleaved by matrix metalloproteinase-9 (MMP-9) and both activity and expression of the protease are known to be reduced by H₂S administration (Du et al., 2017). It has also been reported that MMP-7 can cleave chondroitin sulfate (Lipowsky, 2011) and syndecans 1 and 4 (Manon-Jensen et al., 2013) and our unpublished data indicate that NaGYY4137 inhibits MMP-7 in *in vitro* assays at concentrations similar to those used in the studies described here. Interestingly, our additional preliminary data indicating that NaGYY4137 reduces shedding of hyaluronic acid from cultured ECs suggest another potential action of H₂S since the significant reduction in EC glycocalyx observed in acute hyperglycemia (Nieuwdorp et al., 2006b) and in types 1 and type 2 diabetes (Nieuwdorp et al., 2006a; Broekhuizen et al., 2010) is thought to be related to increased hyaluronidase catabolism (Nieuwdorp et al., 2006a).

It is interesting to note that NaGYY4137 treatment in NG enhanced glycocalyx staining above the levels observed in NG alone (Figure 2), suggesting that H₂S supplementation alone increased glycocalyx density. As mentioned previously, the glycocalyx is a dynamic structure and thus this observation could potentially be explained by the ability of H₂S to modify glycocalyx-degrading enzymes to reduce their activity. Interestingly, H₂S is the only molecule known to induce protein S-persulfidation (S-sulfhydration), a recently identified and unique post-translational protein modification. It is possible that the activity of glycocalyx-degrading enzymes such as heparinase might be altered by persulfidation of the cysteine residues of its active site. Additionally, cysteine residues in proteins have the potential to be redox modified by H₂S in a non-S-persulfidation manner and several potential glycocalyx-degrading enzymes, including the proteinases MMP-1 (Lohakul et al., 2021), MMP-2, MMP-7, and MMP-9, have a cysteine in their active site. Thus, as has been shown with other zinc proteases including angiotensin-converting enzyme (Laggner et al., 2007) and TNF- α -converting enzyme (Li et al., 2013), H₂S has the potential to modulate the glycocalyx-degrading enzyme activity of the proteinases, although further work is needed to confirm this hypothesis.

The effectiveness of our mitochondrial targeted H₂S donor in decreasing retinal leakage *in vivo* raises the possibility of mitochondrial involvement in the observed effects. Modis et al. reported that endogenous mitochondrial H₂S production was governed by MST (Módis et al., 2013). Additionally, Si et al. (2013) reported that DR was associated with significant decreases in retinal H₂S levels and expression of the H₂S synthesizing enzymes CSE, CBS, and 3MST (i.e., DR resulted in retinal H₂S deficiency) and also with increased mitochondrial permeability and respiration (Si et al., 2013), strongly suggesting extensive mitochondrial dysfunction and H₂S deficiency in the retina in DR. Vascular mitochondrial impairment induced by diabetes has previously been shown to be inhibited/reversed by sulfide administration albeit at high doses/concentrations (e.g., 300 μ mol/L) (Módis et al., 2013; Coletta et al., 2015), suggesting that pharmacological sulfide could overcome impaired retinal bioenergetics in DR. Although NaSH administration has previously been shown to reduce retinal vascular abnormalities associated with DR and “correct” mitochondrial dysfunction (Si

et al., 2013), this earlier study is intriguing for several reasons. Firstly, the NaSH was given as a daily intraperitoneal injection (over 14 weeks) at a dose equivalent to 280 mmol/kg. This was an exceptionally high and surprisingly non-lethal dose (although toxicity was not examined) given that 14 μ mol/kg i.p. induces systemic inflammation and vascular collapse (Li et al., 2005) and the LD₅₀ for NaSH is 52.6 μ mol/kg for the same route of administration (Strickland et al., 2003). Possible toxicological constraints aside, the half-life of bolus sulfide in blood is less than a minute as it is rapidly metabolized/removed (Wang, 2002, 2012), so the precise mechanisms by which a bolus of H₂S (from NaSH), administered intraperitoneally survives intact to penetrate the blood–brain barrier and selectively increase sulfide levels in the retina *via* this route remain elusive. Given that plasma H₂S levels have been measured at nanomolar to low micromolar concentrations, our approach to prevent/reverse DR-induced retinal vascular leakage using slow-release H₂S donors (Wei et al., 2014; John et al., 2017; Qiu et al., 2018) or H₂S delivery molecules that selectively target mitochondria (Le Trionnaire et al., 2014; Szczesny et al., 2014; Ikeda et al., 2015; Ahmad et al., 2016; Karwi et al., 2017) would overcome these concerns.

CONCLUSION

The data presented here together with previous *in vivo* data (Whiteman et al., 2010a) suggest that, in health, H₂S is vasculoprotective and that microvascular dysfunction in diabetes, including DR, is associated with reduced circulating and retinal H₂S levels. Thus, diabetes may be a condition of H₂S deficiency and H₂S supplementation using slow-release and/or mitochondrial-targeted H₂S delivery molecules may represent a novel, cost-effective alternative therapy for DR.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Nottingham Biological Services Unit.

AUTHOR CONTRIBUTIONS

JW, DB, and MWh conceived and planned the experiments. CA, JW, DB, and MWh took the lead in writing the manuscript. CA, KW, and NM carried out the experiments. RT and MWO synthesized the slow-release hydrogen sulfide donors. AB and WA contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Conflict of Interest: MWh, RT, and MWO, and the University of Exeter have intellectual property (patent filings) related to hydrogen sulfide delivery molecules and their therapeutic use. MWh was a consultant to MitoRx Therapeutics (Oxford).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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