Effects of Hydrogen Sulfide-Releasing Compounds on Aqueous Humor Outflow Facility in Porcine Ocular Anterior Segments, *Ex Vivo*

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Abstract

Purpose: To investigate the pharmacological actions of hydrogen sulfide (H₂S)-releasing compounds L-cysteine and sodium hydrosulfide (NaHS) on aqueous humor (AH) outflow facility in porcine ocular anterior segment.

Methods: Porcine ocular anterior segments were perfused with Dulbecco's modified Eagle's medium at a constant pressure of 7.35 mmHg. After stable outflow baseline, explants were exposed to NaHS or L-cysteine. The increase in outflow generated by the H₂S-releasing compounds was measured in the absence and presence of inhibitor of H₂S biosynthesis (aminooxyacetic acid; AOAA), blocker of K_{ATP} channels (glibenclamide), and inhibitor of adenylyl cyclase (SQ 22536). Hematoxylin and eosin (H&E) staining was used to assess trabecular meshwork (TM) morphology.

Results: L-cysteine elicited a concentration-dependent increase in AH outflow facility, reaching maximal effect at 100 nM (150.6% ± 17.2% of basal level). This increase in outflow induced by L-cysteine was significantly ($P < 0.001$) antagonized by AOAA (30 μM) and glibenclamide (100 μM). AOAA and glibenclamide had no significant action on baseline outflow, whereas SQ 22536 (100 μM) increased outflow for only an hour. In addition, NaHS produced a concentration-dependent increase in AH outflow, with a maximal effect at 10 μM (151.4% ± 22.9% of basal level). Likewise, the increase in outflow caused by NaHS was significantly ($P < 0.04$) blocked by glibenclamide and SQ 22536. H&E staining revealed that L-cysteine or NaHS did not alter TM conformation.

Conclusion: H₂S-releasing compounds can increase outflow facility in porcine ocular anterior segment. The stimulatory action of these compounds on outflow is mediated, in part by endogenously produced H₂S, K_{ATP} channels, and adenylyl cyclase.

Keywords: hydrogen sulfide, aqueous humor outflow, trabecular meshwork

Introduction

HYDROGEN SULFIDE (H₂S) has recently emerged as a new gaseous mediator of biological relevance in mammalian tissues.^{1–3} H₂S is endogenously produced in mammalian tissues by the desulfurization of L-cysteine catalyzed by 3 enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST).⁴ In the eye, the 3 enzymes responsible for the biosynthesis of H₂S (CBS, CSE, and 3-MST) have been localized in tissues such as the cornea, iris, ciliary body, lens, and retina.^{5,6} There is evidence that a

deficiency of CBS because of a mutation in the gene encoding the enzyme can lead to ocular disorders such as glaucoma, lens dislocation, and retinal detachment.⁷ Indeed, the concentrations of homocysteine, a substrate for the biosynthesis of H₂S, have been reported to be elevated in the tear fluid, plasma, and aqueous humor (AH) of patients with primary open-angle glaucoma.⁸ Evidence from our laboratory demonstrates that H₂S can exert pharmacological actions on ocular tissues such as the relaxation of smooth muscles, and the inhibition of neurotransmitter release from the anterior uvea and retina.^{9–11} Furthermore, we have reported that H₂S is endogenously produced in isolated mammalian ocular tissues

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and can increase cyclic adenosine monophosphate (AMP) concentrations in the retina.^{12–14} Other investigators have demonstrated an intraocular pressure (IOP)-lowering action of ACS67, a H₂S-releasing molecule in glaucomatous rabbits, and the ability of sodium hydrosulfide (NaHS) to protect rat retinal neurons from light-induced retinal degeneration and N-methyl-D-aspartate-induced neuronal injury.^{6,15,16} In summary, H₂S appears to have physiological, pathological, and pharmacological roles in the eye.

Although H₂S-releasing compounds have been reported to lower IOP in normotensive and experimental animals,^{12,17} their exact mechanism of action is unknown. The aim of this study was 2-fold: (a) to investigate the pharmacological actions of L-cysteine, the substrate for biosynthesis of H₂S and NaHS, and a H₂S-releasing compound on AH outflow facility in a porcine ocular anterior segment model, *ex vivo*, and (b) to examine the potential mechanisms involved in the observed changes elicited by compounds that increase H₂S availability on the outflow facility. Parts of the data presented this article have been communicated in a preliminary form as abstracts.^{18,19}

Methods

Chemicals

L-cysteine, NaHS, glibenclamide, SQ 22536, and aminooxyacetic acid (AOAA) were all purchased from Sigma Aldrich, St. Louis, MO. Stock solutions of SQ 22536 were dissolved in dimethyl sulfoxide. Stock solutions of all other test compounds were made immediately before use in distilled water. Just before perfusion, drugs were dissolved in Dulbecco's modified Eagle's medium (DMEM) to final concentrations. Vehicle controls were also prepared daily using 0.1% saline in DMEM.

Perfusion of ocular anterior segments treated with H₂S donors

Fresh porcine eyes were obtained from Vision Tech (Dallas, TX) and transported to the laboratory on ice, within 18 h after decapitation. Eyeballs were not subjected to additional treatment after enucleation other than being preserved in an ice bath. The methodology utilized in setting up the porcine ocular anterior segment perfused organ culture model is essentially the same as described by Zhong et al.²⁰ and Bradley et al.²¹ Briefly, porcine ocular anterior segment explants were dissected by carefully removing the iris and ciliary muscle, thus leaving only the trabecular meshwork (TM), cornea, and small (5 mm) rim of sclera. Explants were then mounted in the ocular perfusion culture chambers and perfused with DMEM for 3–4 h using a constant-perfusion head set at a height of 10 cm for a constant pressure of 7.35 mm Hg. Only explants with an outflow baseline rate from 1.5 to 6 μ L/min at a pressure of 7.35 mm Hg were deemed stable and subsequently employed in experiments. Explants that do not reach a stable baseline within this time period (3–4 h) are excluded from experiments. It has been previously demonstrated that in this model, outflow is through the TM, and flow rates are physiological ($\sim 2.75 \mu$ L/min).²¹ Following stable baseline perfusion rates, NaHS or L-cysteine was administered into the perfusion chambers. Determination of outflow facility began 30 min after drug application, and was measured at an in-

terval of 1 h, for a total of 4 h. In experiments when AOAA (30 μ M) (inhibitor of the CBS and CSE, enzymes for biosynthesis of H₂S), glibenclamide (100 μ M) (blocker of K_{ATP} channels), and SQ 22536 (100 μ M) (inhibitor of adenylyl cyclase) were used, ocular segments were perfused with these agents for 30 min before exposure to NaHS or L-cysteine. Drug concentrations chosen for this study are based on maximally effective concentrations as evident from the literature and previous work from our laboratory. Vehicle controls (0.1% saline) were run in parallel for each group of experiments. At the end of the perfusion studies, anterior segments were removed from the perfusion chambers, and 2–3-mm-wide wedges from each quadrant containing outflow tissues were cut and submerged in 10% formalin overnight, and forwarded to the Histology Core at Baylor College of Medicine, Houston, Texas, for hematoxylin and eosin (H&E) staining and evaluation by light microscopy. Perfusion studies were considered invalid and excluded if anterior segments had morphological changes consistent with autolysis.

Data analysis

Outflow facility was calculated as the ratio of the rate of flow of perfusate (μ L/min) to the steady state perfusion pressure (7.35 mmHg). Effects of L-cysteine and NaHS on outflow facility were assessed in each eye as percentage change of outflow facility in treated eyes over pretreatment baseline outflow. Between 6 and 12 porcine eyes were used for each concentration treatment group. Data are represented as the mean $\pm$ standard error, plotted as change in outflow facility with respect to time, and were analyzed using

FIG. 1. Effects of L-cysteine on AH outflow facility in porcine. Porcine anterior segments were treated in perfusion culture with 3 concentrations of L-cysteine (1 nM–1 μ M). Control eyes were treated with 0.1% saline prepared in DMEM. The 1 and 100 nM concentrations produced a statistically significant change in outflow compared with vehicle, whereas 1 μ M concentration had no significant effect. Results are depicted as the mean $\pm$ SE, $n = 8$. *Significant difference between L-cysteine treated and 0.1% saline vehicle ($P < 0.05$). AH, aqueous humor; DMEM, Dulbecco's modified Eagle's medium; SE, standard error.

GraphPad software. Increase in outflow facility compared with vehicle was deemed statistically significant if $P < 0.05$ using ANOVA and paired Student *t*-test.

Results

Effects of L-cysteine and NaHS on outflow facility

Both L-cysteine and NaHS caused a concentration-dependent increase in outflow facility from perfused porcine ocular anterior segments, *ex vivo*. Figure 1 illustrates that the effects of L-cysteine (1 nM–1 μ M) reached a peak, reflecting $151\% \pm 17\%$ ($n=8$) increase in outflow facility over basal levels ($99\% \pm 7.3\%$) at 100 nM ($P < 0.04$). In addition, L-cysteine (1 nM) significantly increased outflow facility for a maximal effect 1 h after administration, $133.7\% \pm 16.3\%$ of basal level ($99\% \pm 7.3\%$; $P < 0.03$). Neither the vehicle (0.1% saline) nor L-cysteine (1 μ M) had any significant effect on baseline outflow facility. Likewise, NaHS (10 nM–10 μ M) produced a concentration-related increase in outflow facility with 10 μ M of this compound eliciting $151\% \pm 23\%$ ($n=8$) change over baseline outflow facility ($98.8\% \pm 8\%$; $P < 0.02$; Fig. 2). Neither the vehicle (0.1% saline) nor concentrations of NaHS lower than 10 nM had any significant effect on baseline outflow facility.

Effect of AOAA on L-cysteine-induced enhancement of outflow facility

To investigate the role of endogenous enzymes for the biosynthesis of H₂S in the response elicited by L-cysteine, we added an inhibitor of CBS/CSE (AOAA, 30 μ M) to the perfusion medium before exposure of explants to L-cysteine (100 nM). As shown in Fig. 3, although AOAA had no significant effect ($P > 0.05$) on outflow facility (Fig. 3B), it blocked significantly ($P < 0.05$) the L-cysteine-mediated increase in

FIG. 2. Effects of NaHS on AH outflow facility in porcine. Porcine anterior segments were treated in perfusion culture with 3 concentrations of NaHS (10 nM–10 μ M). Control eyes were treated with 0.1% saline prepared in DMEM. The 10 μ M concentration produced a statistically significant change in outflow than that of the vehicle. Results are depicted as the mean $\pm$ SE, $n=8$. *Significant difference between NaHS treated and 0.1% saline vehicle ($P < 0.05$).

FIG. 3. Antagonism of L-cysteine-induced increase of AH outflow facility by AOAA. The porcine anterior segments were treated with 30 μ M AOAA for 30 min before the addition of 100 nM L-cysteine (A). Effects of AOAA on basal outflow facility (B). Results are expressed as the mean $\pm$ SE; $n=8$. *Significant differences between L-cysteine alone and L-cysteine plus AOAA ($P < 0.05$). AOAA, aminooxyacetic acid.

outflow facility from a maximal effect of $151\% \pm 17\%$ ($n=8$) to $106\% \pm 10\%$ ($n=8$) of baseline outflow facility (Fig. 3A).

Effect of glibenclamide on L-cysteine- and NaHS-induced enhancement of outflow facility

As there is evidence that the pharmacological actions of H₂S-releasing compounds are mediated by K_{ATP} channels,^{22,23} we next examined the effect of glibenclamide, a blocker of K_{ATP} channels, on responses produced by L-cysteine and NaHS. Prior exposure of explants to glibenclamide (100 μ M) had no significant action on baseline outflow facility (Fig. 4C). As shown in Fig. 4A and B, glibenclamide pretreatment blocked significantly L-cysteine (100 nM; $P < 0.005$)- and NaHS (10 μ M; $P < 0.05$)-induced enhancement of outflow facility over baseline levels, respectively.

Effect of SQ 22536 on NaHS-induced increase in outflow facility

Njie-Mbye et al. reported that cyclic AMP mediates the pharmacological actions of H₂S-releasing compounds in isolated bovine and porcine neural retinae and rat retinal pigment epithelial cells.^{13,14} Consequently, we designed experiments to investigate the role of cyclic AMP in the responses produced by NaHS on outflow facility. The

FIG. 4. Inhibition of L-cysteine- and NaHS-induced increase of AH outflow facility by the K_{ATP} blocker glibenclamide (Glib). The porcine anterior segments were treated with 100 μ M glibenclamide for 30 min before the addition of 100 nM L-cysteine (A) or 10 μ M NaHS (B). Effects of Glib on basal outflow facility (C). Results are expressed as the mean $\pm$ SE; $n=8$. *Significant differences between L-cysteine or NaHS alone and L-cysteine/NaHS plus glibenclamide ($P<0.05$).

adenylyl cyclase inhibitor, SQ 22536 (100 μ M), increased outflow facility ($P<0.01$) for only 1 h before returning to baseline (Fig. 5B). Pretreatment of explants with SQ 22536 blocked significantly ($P<0.05$) the NaHS (10 μ M)-induced increase in outflow facility (Fig. 5A).

Effects of NaHS and L-cysteine on TM morphology

To determine whether H_2S -releasing compounds had any effect on TM morphology, ocular segments were evaluated by light microscopy after H&E staining. Previous reports have shown that the outflow pathway tissues are intact and viable after 5 h of perfusion with DMEM or vehicle (0.1% saline).^{24,25} As illustrated in Fig. 6C and D, the controls (0.1% saline and DMEM) had no effect on TM viability. Vehicle- (0.1% saline) and DMEM-treated ocular explants displayed normal morphology of the TM, angular aqueous plexus, and sclera with no changes suggestive of ocular hypertension. Likewise, the 10 μ M NaHS-mediated increase in outflow facility did not affect the TM outflow pathway (Fig. 6A). Similar to vehicle control, 100 nM L-cysteine-treated segments of TM showed no changes suggestive of ocular hypertension and/or autolytic change (Fig. 6B). Outflow pathway tissues were intact and contained open spaces consistent with the angular aqueous plexus.

Discussion

It is now well documented that the enzymes responsible for the biosynthesis of H_2S are present in tissues and organs of several mammalian species, indicating that this gas may have a biological role in health and disease states. In the eye, CBS, CSE, and 3-MST have been localized in tissues of both the anterior and posterior segments.^{5,6,8} Evidence from our laboratory demonstrates that H_2S can be produced in

bovine ocular tissues such as the cornea, iris, ciliary body, lens, retina, and choroid but not in the vitreous humor.¹² The presence of enzymes responsible for the biosynthesis of H_2S in anterior uveal tissues coupled with the production of this gas indicates that the pathways associated with H_2S metabolism may be involved in the regulation of AH dynamics. Indeed, concentrations of homocysteine, a substrate for the biosynthesis of H_2S , have been reported to be elevated in the tear fluid, plasma, and AH of 85% of the patient population with primary open-angle glaucoma.⁸ In earlier studies, we reported that H_2S -releasing compounds can exert pharmacological actions on tissues of the anterior uvea such as in causing the relaxation of iris smooth muscles^{10,26} and inhibition of norepinephrine release from sympathetic nerves.⁹ In the retina, we found that although H_2S -releasing compounds can inhibit D-aspartate release,¹¹ they can also increase cyclic AMP concentrations in isolated bovine and porcine tissues and in rat retinal pigment epithelial cells.^{13,14} In a recent study, we found that H_2S -releasing compounds can lower IOP in normotensive rabbits.¹⁷ Likewise, in 2009, Perrino et al. demonstrated that ACS67, a H_2S -releasing molecule, can lower IOP in glaucomatous rabbits.¹⁵ Taken together, these observations support a physiological, pathological, and pharmacological role for H_2S in the anterior segment of the eye.

Tissues of the TM and Schlemm's canal regulate and maintain the homeostatic response to IOP. Under normal physiological conditions, the trabecular outflow pathways play a major role in the generation and maintenance of IOP because they provide resistance against the flow of AH.^{27,28} Studies of the regulation of AH from conventional outflow channels have utilized anterior segment perfused organ cultures from humans and animals.^{20,29} Utilization of the porcine anterior ocular perfusion model allows evaluation of drugs on AH dynamics without affecting the conventional

FIG. 5. Blockade of NaHS-induced increase of AH outflow facility by the adenylyl cyclase inhibitor SQ 22536 (SQ). The porcine anterior segments were treated with 100 μ M SQ 22536 for 30 min before the addition of 10 μ M NaHS (A). Effects of SQ on basal outflow facility (B). Results are expressed as the mean $\pm$ SE; $n=8$. *Significant differences between SQ 22536 or NaHS alone and NaHS plus SQ 22536 ($P<0.05$).

pathway composition and protection from external biological changes.^{29,30} In this study, we examined the effect of L-cysteine, a substrate for the biosynthesis of H₂S and NaHS, a fast donor of H₂S, on outflow facility in the porcine ocular anterior segment model, *ex vivo*. We observed that both the

substrate for the biosynthesis of H₂S and a fast donor of H₂S produced a concentration-dependent increase in the outflow facility. Furthermore, the outflow effect of the substrate, L-cysteine, was biphasic. It is well reported that H₂S-releasing compounds can elicit a biphasic response.^{31–33} This biphasic response may be caused by an action of L-cysteine on more than one pathway or that these pathways are active at different concentrations of L-cysteine. To the best of our knowledge, this is the first demonstration of an effect of compounds that increase H₂S availability on channels that play a major role in the outflow of AH. It is tempting to speculate that the observed increase in outflow facility may account, at least in part, for the ability of H₂S-releasing compounds to lower IOP as has been reported by our group and others.^{15,17}

As a substrate, L-cysteine has to be converted to H₂S through a process catalyzed by enzymes such as CBS, CSE, or 3-MST.^{4,34} Recently, AOAA has been shown to inhibit both CBS and CSE in biological tissues.³⁵ In this study, we found that AOAA (30 μ M) blocked the increase in outflow facility induced by L-cysteine in the porcine ocular anterior segments, *ex vivo*. On its own, AOAA had no significant action on baseline outflow facility in the explants. A similar blockade of the pharmacological actions of L-cysteine by AOAA has been reported by other investigators.^{12,13} These results indicate that the observed pharmacological effects of L-cysteine on outflow facility could be because of the *in situ* biosynthesis of H₂S, which, in turn, elicits the response.

The pharmacological actions of H₂S have been ascribed to an effect on ATP-sensitive potassium channels (K_{ATP}) in the cardiovascular, central nervous systems, and eye.^{23,36,37} In this study, we investigated the effect of glibenclamide, a blocker of K_{ATP} channels, on L-cysteine- and NaHS-induced increase in outflow facility in the porcine anterior segment explants, *ex vivo*. It is pertinent to note that in addition to inhibition of K_{ATP} channels, glibenclamide has additional modes of action.^{38,39} In this study, glibenclamide blocked the increase in outflow facility induced by both L-cysteine and NaHS without affecting baseline flow. Indeed, glibenclamide has also been shown to antagonize the pharmacological actions of H₂S-releasing compounds in several biological systems.⁴⁰ Our results support the view that H₂S-releasing compounds may

FIG. 6. Morphology of the TM from perfusion-cultured porcine anterior segments. Tissues were imbedded in paraffin, sectioned, and stained with H&E. The TM of a porcine eye after perfusion culture with 10 μ M NaHS (A), 100 nM L-cysteine (B), DMEM (C), and 0.1% saline (D). AAP, aqueous angular plexus; H&E, hematoxylin and eosin; S, sclera; TM, trabecular meshwork.

exert their pharmacological actions on AH outflow pathways through K_{ATP} channels.

Cyclic AMP has been implicated in several neuronal functions, including axon regeneration, modulation of axonal guidance, cell survival, and in the regulation of AH secretion and outflow.^{41,42} Kimura showed that exogenously administered H_2S increased the generation of cyclic AMP in primary cultures of rat cerebral and cerebellar neurons, and in some neuronal and glial cells.⁴³ Evidence from our laboratory demonstrates that H_2S -releasing compounds can increase cyclic AMP production in isolated bovine and porcine neural retinae and in rat retinal pigment epithelial cells.^{13,14} Based on the role of cyclic AMP in the regulation of AH secretion and outflow and the reported actions of H_2S -releasing compounds on the production of this nucleotide in several tissues and cells, it was of interest to examine the role of cyclic AMP in the pharmacological effect of NaHS on outflow facility in the porcine ocular anterior segment. The adenylyl cyclase inhibitor, SQ 22536, abolished the increase in outflow facility induced by NaHS and elicited an initial increase in baseline flow that lasted for only 1 h. The lack of a measurable response by SQ 22536 on baseline outflow at the subsequent time points may be caused by a low level of basal cAMP concentration in the explants. Indeed, it has been reported by others that basal cyclic AMP is low in the TM.^{44,45} These results strongly suggest that the pharmacological effects of NaHS on outflow facility could be dependent, at least in part, on the production of cyclic AMP.

Using light microscopy, we uncovered no differences in the morphology of the TM between L-cysteine and NaHS when compared with tissues treated with 0.1% saline and DMEM. Our findings support the view that the anterior perfusion culture chamber is a good model for studying AH outflow because it maintains the delicate TM anatomy and is not affected by biological changes occurring outside of the chamber. Our data are similar to those from other studies utilizing similar perfusion models, which saw no morphological changes to the primary outflow pathway after perfusion lasting up to 9 days.^{24,46}

We conclude that compounds that can increase the availability of H_2S such as L-cysteine and NaHS can increase outflow facility in a porcine ocular anterior segment model, *ex vivo*. The ability of the H_2S substrate, L-cysteine, to increase outflow facility is dependent, at least in part, on *in situ* biosynthesis of H_2S . K_{ATP} channels are involved in the pharmacological actions of H_2S -releasing compounds on outflow facility. The pharmacological effect of NaHS on outflow facility in the porcine ocular anterior segment is dependent, at least in part, on adenylyl cyclase activity. The exact mechanisms that underlie the pharmacological actions of H_2S -releasing compounds on signal transduction pathways involved in the regulation of AH outflow channels are unknown and merit further investigation. Our recent report of an IOP-lowering effect of H_2S -releasing compounds¹⁷ and our findings in this study of an increased AH outflow effects by these agents are of great interest and merits for us to validate our observed H_2S outflow effects in a living animal model.

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Author Disclosure Statement

No competing financial interests exist.

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