Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function


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The goal of the present studies was to investigate the role of changes in hydrogen sulfide (H2S) homeostasis in the pathogenesis of hyperglycemic endothelial dysfunction. Exposure of bEnd3 microvascular endothelial cells to elevated extracellular glucosel (in vitro hyperglycemia) induced the mitochondrial formation of reactive oxygen species (ROS), which resulted in an increased consumption of endogenous and exogenous H2S. Replacement of H2S or overexpression of the H2S-producing enzyme cystathionine-γ-lyase (CSE) attenuated the hyperglycemia-induced enhancement of ROS formation, attenuated nuclear DNA injury, reduced the activation of the nuclear enzyme poly(ADP-ribose) polymerase, and improved cellular viability. In vitro hyperglycemia resulted in a switch from oxidative phosphorylation to glycolysis, an effect that was partially corrected by H2S supplementation. Exposure of isolated vascular rings to high glucose in vitro induced an impairment of endothelium-dependent relaxations, which was prevented by CSE overexpression or H2S supplementation. siRNA silencing of CSE exacerbated ROS production in hyperglycemic endothelial cells. Vascular rings from CSE−/− mice exhibited an accelerated impairment of endothelium-dependent relaxations in response to in vitro hyperglycemia, compared with wild-type controls. Streptozotocin-induced diabetes in rats resulted in a decrease in the circulating level of H2S; replacement of H2S protected from the development of endothelial dysfunction ex vivo. In conclusion, endogenously produced H2S protects against the development of hyperglycemia-induced endothelial dysfunction. We hypothesize that, in hyperglycemic endothelial cells, mitochondrial ROS production and increased H2S catabolism form a positive feed-forward cycle. H2S replacement protects against these alterations, resulting in reduced ROS formation, improved endothelial metabolic state, and maintenance of normal endothelial function.

H2S Replacement Exerts Cytoprotective Effects in Hyperglycemic Endothelial Cells in Vitro. Addition of H2S (100–300 μM) for the last 4 d of the 7-d hyperglycemic period provided a concentration-dependent protection against cellular ROS production (Fig. 2 A and B) and attenuated mitochondrial membrane depolarization as measured by the fluorophore 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzamidazol-carbocyanine (JC-1) (Fig. 2 C). Exposure of the cells to intermittent high/low-glucose conditions is known to exacerbate hyperglycemic endothelial dysfunction (18–20). Accordingly, intermittent high/low glucose induced a more pronounced increase in ROS production compared with a steady elevation of glucose, and H2S continued to attenuate this response (Fig. 2 D). H2S administration also reduced DNA fragmentation (Fig. S14) and the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Fig. S1F), which are known downstream consequences of mitochondrial ROS formation in this response (Fig. 2 D).

Results

In Vitro Hyperglycemia Is Associated with Increased H2S Degradation Caused by Mitochondrial ROS Overproduction. Exposure of endothelial cells to elevated glucose for 7 d resulted in a significant suppression of the H2S concentration in the supernatant (Fig. 1A). Incubation of the cells with the ROS scavenger Tempol increased the H2S concentration in the medium (Fig. 1B), suggesting that the reduced H2S level in hyperglycemia may be attributable to increased consumption of H2S by ROS. Administration of H2S to culture medium (without cells) resulted in a decline of ambient H2S concentrations because of the reaction of H2S with oxygen and culture media constituents. The consumption of H2S was increased in the presence of normoglycemic cells and was further accelerated in the presence of hyperglycemic cells (Fig. 1B). Addition of the mitochondrial uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to hyperglycemic cells resulted in a slower consumption rate of H2S (Fig. 1B), consistent with the hypothesis that mitochondrially derived ROS production contributes to the increase in H2S consumption in hyperglycemic cells. The increased mitochondrial ROS production in hyperglycemic cells was demonstrated by the redox-sensitive dye MitoSOX red; ROS production was reduced by the uncoupling agents CCCP and thenoyltrifluoracetone (TTFA) (Fig. 1C), confirming previous studies (11–14) showing that mitochondria represent a major source of ROS.

H2S Replacement Exerts Cytoprotective Effects in Hyperglycemic Endothelial Cells in Vitro. Addition of H2S (100–300 μM) for the last 4 d of the 7-d hyperglycemic period provided a concentration-dependent protection against cellular ROS production (Fig. 2A and B) and attenuated mitochondrial membrane depolarization as measured by the fluorophore 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzamidazol-carbocyanine (JC-1) (Fig. 2C). Exposure of the cells to intermittent high/low-glucose conditions is known to exacerbate hyperglycemic endothelial dysfunction (18–20). Accordingly, intermittent high/low glucose induced a more pronounced increase in ROS production compared with a steady elevation of glucose, and H2S continued to attenuate this response (Fig. 2D). H2S administration also reduced DNA fragmentation (Fig. S14) and the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Fig. S1F), which are known downstream consequences of mitochondrial ROS formation in this response (Fig. 2D).


Conflict of interest statement: C.S. is a stockholder in Ikaria Inc., a for-profit organization involved in the development of H2S-based therapeutics. This article is PNAS Direct Submission.

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Overexpression of CSE in endothelial cells elevated extracellular H2S levels in normoglycemic cells (by 32 ± 5%), but only to a smaller degree in hyperglycemic cells (by 10 ± 3%), consistently with the increased consumption of H2S during hyperglycemia, as demonstrated above. Overexpression of CSE attenuated the hyperglycemia-induced increase in ROS production (Fig. 3A). Both pharmacological replacement of H2S and overexpression of CSE protected against the hyperglycemia-induced decline in cellular viability. For instance, hyperglycemia decreased cell viability by 18 ± 2% (P < 0.05), whereas in the endothelium overexpressing CSE, cell viability increased by 6 ± 3%, compared with normoglycemic controls (n = 4).

Mechanisms of the Cytoprotective Effect of H2S in Hyperglycemic Endothelial Cells. Analysis of the cellular metabolic status of the cells showed that high glucose induces a shift away from the mitochondrial oxidative phosphorylation toward glycolysis: the basal respiratory capacity and the respiratory reserve capacity response to carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) were reduced in hyperglycemic cells, compared with the normoglycemic cells (Fig. 4). Treatment of the cells with H2S resulted...
in an improvement of mitochondrial respiration, whereas the hyperglycemia-induced increase in the glycolytic activity of the cells was normalized (Fig. 4). There were no marked alterations in the cellular ATP levels in hyperglycemic cells, likely because of the compensatory effect of increased glycolysis (ATP levels decreased to 88 ± 1% in hyperglycemic endothelial cells vs. normoglycemic endothelial cells; n = 3, P < 0.05). H2S prevented the hyperglycemia-induced suppression of cellular ATP levels (104 ± 2% of control normoglycemic cells, n = 3). Thus, H2S treatment produced a partial reversal of the hyperglycemia-induced metabolic switch and normalized the energetic status of the cells. As opposed to the effects of H2S administration for 4 d (see above), a short period of H2S administration (the last 60 min of the hyperglycemic period) failed to affect the mitochondrial ROS production (Fig. S2). Thus, although high concentrations of H2S are known to inhibit mitochondrial cytochrome c oxidase, resulting in a suppression of mitochondrial oxidative phosphorylation (21–23), the present effects of H2S in decreasing mitochondrial ROS production in hyperglycemic endothelial cells are not mediated by an acute suppression of mitochondrial function.

The expression of CSE (a principal H2S-producing enzyme in vascular tissues) was not affected by exposure of the endothelial cells to elevated glucose for 7 d (Fig. 2A). Furthermore, the effects of H2S in hyperglycemic endothelial cells did not depend on the activation of the ATP-sensitive potassium (KATP) channel because the KATP channel blocker glibenclamide failed to reverse the protective effect of H2S (Fig. S3).

**Discussion**

The formation of ROS from endothelial cells is a key factor in the pathogenesis of diabetic complications (11, 12). In addition, increased ROS formation and endothelial dysfunction has been linked to various forms of critical illness, to postoperative conditions, as well as to impaired glucose tolerance conditions and postprandial hyperglycemia (11, 12, 24–31). Mitochondrial electron transport is recognized as a key source of ROS in hyperglycemic endothelial cells (11, 12). ROS, on their own and by combining with endothelial nitric oxide to form the reactive oxidant peroxynitrite, can induce DNA damage and activation of suicidal pathways governed by the nuclear enzyme PARP (32).

The biosynthesis of H2S, as well as the biological degradation (consumption) of H2S, is a dynamic process (3, 5, 15). The current results point to the existence of a crucial interplay between endothelial H2S formation and ROS production in maintaining mitochondrial function: elevated glucose perturbs this balance. Our data demonstrate that the consumption of H2S is accelerated in endothelial cells placed in elevated glucose, an effect that depends on mitochondrial ROS formation (because it can be attenuated by mitochondrial uncoupling). It is conceivable that this accelerated H2S consumption is responsible for the lower baseline levels of H2S detected in the medium of cells placed in elevated extracellular glucose and for the decreased H2S levels measured in the circulation of streptozotocin-diabetic rats. On the other hand, down-regulation of CSE does not occur in hyperglycemia and diabetes under our experimental conditions and, therefore, is not responsible for the reduced H2S levels.

H2S, as a reducing agent and an antioxidant molecule, has been previously shown to protect various cell types from oxidative injury (33–36). Based on the current results, we hypothesize that H2S provides a reducing/antioxidant intracellular environment that contributes to the maintenance of normal mitochondrial function. This balance is perturbed when mitochondrial ROS production is stimulated by high concentrations of glucose. We hypothesize that the ROS from hyperglycemic mitochondria directly reacts with and consumes the intracellular H2S, which then creates additional mitochondrial dysfunction, possibly by oxidative modification to mitochondrial proteins. Such a positive feed-forward cycle may then culminate in a dysfunctional mitochondrial state where molecular oxygen is used to produce ROS (as opposed to ATP) and where mitochondrial efficacy is diminished. These events will lead to a loss of mitochondrial membrane potential and, finally, a spillage of ROS to the cytosolic and nuclear compartments.
Recent studies have demonstrated that antioxidant depletion is a hallmark of hyperglycemia in endothelial cells (37–39). It has also been demonstrated previously that endothelial ROS overproduction leads to oxidative and nitrosative protein modifications, DNA injury, and activation of secondary deleterious cellular cycles of injury, such as the one governed by the activation of PARP (11–14, 18–20, 32). The beneficial effects of antioxidants on the endothelial function in hyperglycemia may be, at least in part, related to the preservation of the endothelial H2S homeostasis.

The current bioenergetic findings, in agreement with a recent analysis of bioenergetic alterations in rat retinal endothelial cells placed in high extracellular glucose (40), demonstrate that bEnd3 endothelial cells placed in high extracellular glucose exhibit a reduced oxygen consumption rate (i.e., reduced mitochondrial oxidative phosphorylation), an effect that is partially counterbalanced by an up-regulation of glycolysis. Our results also demonstrate that H2S replacement therapy protects against this pathophysiological switch between oxidative phosphorylation and glycolysis. We conclude that restoration of oxidative phosphorylation, coupled with an improvement of cellular ATP levels, mitochondrial depolarization, and mitochondrial ROS production are the key intracellular events through which H2S replacement is able to restore normal cellular function in hyperglycemic endothelial cells and prevent the development of endothelial dysfunction.

The results of the current study demonstrate that replacement of the H2S, either by supplementation into the culture medium or by overexpression of the H2S-producing enzyme CSE, is able to protect from the deleterious consequences of hyperglycemia. On the other hand, siRNA silencing of CSE, or the deletion of the CSE gene, results in conditions where hyperglycemia induces an exacerbated endothelial response (more ROS production and more severe loss of endothelium-dependent relaxant function), consistent with the hypothesis that endogenous H2S plays a protective role against the deleterious consequences of hyperglycemia in endothelial cells.

Our in vivo/ex vivo observations, showing that supplementation of H2S to streptozotocin-diabetic rats improves the endothelium-dependent relaxant function of vascular rings, are consistent with our in vitro findings in endothelial cells. Also, previous studies have demonstrated that pharmacological interventions (e.g., ROS scavenging, peroxynitrite neutralization, PARP inhibition) that protect hyperglycemic endothelial cells are also able to improve endothelium-dependent relaxations in diabetic rodents (11, 12, 29–32), and, therefore, prevention of the activation of these downstream pathways is likely to contribute to the effects of H2S in the current experimental system. The translational value of the current findings is enhanced by the observation that circulating H2S levels are lowered not only in streptozotocin-diabetic and in NOD mice (a model of type 1 diabetes) but also in patients with diabetes (41–43). We conclude that hyperglycemia produces a H2S-deficient state in endothelial cells: H2S replacement therapy in hyperglycemic conditions may be of therapeutic potential.

Methods

Cell Culture. The bEnd3 microvascular endothelial cell line was purchased from the American Type Culture Collection, cultured at 37 °C at 5% CO2, in a humidified chamber, with 5.5 mM glucose containing DMEM with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% FCS, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1%
H2S levels (* using the H2S-releasing minipumps (STZ/S); (B) The hyperglycemic response is unaffected by H2S-releasing minipumps: *P<0.05 shows significant and comparable degree of hyperglycemia in STZ rats treated with vehicle or H2S-releasing pumps. (C) The thoracic aortas of streptozotocin-diabetic rats (STZ/V) exhibit reduced endothelium-dependent relaxant function in response to acetylcholine (1 nM to 30 μM). (D) The percentage decrease of the relaxation by high glucose between CSE+/+ and CSE−/− mice; *P<0.05 shows a higher degree of impairment of the relaxations in the CSE−/− rings than in wild-type rings (n=6–12).

ROS production induced by alternating (12-h cycle) high/low-glucose conditions. To overexpress CSE in endothelial cells, cells were transfected with an adenoviral plasmid as previously described (44). For CSE silencing, Silencer Select siRNA for CSE and nonsense control siRNA were obtained from Ambion and were transfected with Lipofectamine 2000.

Measurement of H2S Levels. Amperometric H2S sensors (WPI) were used for the real-time measurement of dissolved H2S concentration in the medium (15). The amperometric H2S sensor was calibrated before each experiment with freshly prepared (anoxic) NaHS stock solution (0–300 μM).

Measurement of Mitochondrial ROS Production. MitoSOX red (Invitrogen), a mitochondrion-specific hydroethidine-derivative fluorescent dye, was used to assess mitochondrial O2− production in situ (45).

Western Blotting. Whole-cell lysate were made by using RIPA buffer with EDTA with a protein protease inhibitor mixture. Equal amounts of protein lysate were separated with 8% SDS/PAGE gels, transferred to a 0.45-μm nitrocellulose. The membrane was blocked with 5% low-fat milk in PBS or Tris-buffered saline containing 0.05% Tween-20 and incubated with the primary antibody overnight at 4 °C. Primary antibodies for CSE, CBS, and PARP were from Santa Cruz Biotechnology, and for actin, from Sigma.

Quantitation of DNA Strand Breaks. DNA strand breaks were detected with a single-cell gel electrophoresis assay (14). DNA strand breaks were quantitated by examining the fixed and stained cells under a fluorescence microscope. The mean length of the DNA tail was determined by measuring 20 cells for each condition.

Cell Viability Assay. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to measure mitochondrial respiration, as an indicator of cell viability (46).

Bioenergetic Analysis. The XF24 Analyzer (Seahorse Biosciences) was used to measure bioenergetic function in intact bEnd3 cells. The XF24 creates a transient 7-μL chamber in specialized microplates that allows for oxygen...
consumption rate and extracellular acidification rate to be monitored in real time (40, 47). To measure indices of mitochondrial function, oligomycin, FCCP, and antimycin A were injected sequentially at the level of non-ATP-linked oxygen consumption (proton leak), the maximal respiratory capacity, and the nonmitochondrial oxygen consumption. Cel- lular ATP content was measured by a luminescence assay (48).

**Vascular Studies of in Vitro Hyperglycemia.** Thoracic aortic rings from Sprague-Dawley rats were incubated for 48 h under normoglycemic or hyperglycemic conditions in DMEM as described above, in the presence or absence of 200 μM NaHS, applied every 8 h, followed by the determination of endothelium-dependent relaxations (29). Adenoviral CSE overexpression in vascular rings was performed as described for endothelial cells above, followed by in- dependent relaxations (29). Adenoviral CSE overexpression in vascular rings sequentially at the level of non-ATP-linked oxygen consumption, the amount of oxygen consumption linked to ATP production, the nonmitochondrial oxygen consumption, respectively. Using these agents, we determined the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production, mitochondrial function, oligomycin, FCCP, and antimycin A were injected sequentially at the level of non-ATP-linked oxygen consumption (proton leak), the maximal respiratory capacity, and the nonmitochondrial oxygen consumption. Cell- lular ATP content was measured by a luminescence assay (48).

**Vascular Studies in Diabetic Rats.** Diabetes in male Sprague-Dawley rats was induced with a single streptozotocin injection of 60 mg/kg of body wt i.p. prepared in citrate buffer (pH 4.5). On day 14, animals were implanted with osmotic pumps (Alzet) filled with NaHS (releasing a dose of 16 μg/kg per min) or vehicle. Rats were divided into groups as follows: control group (CTRL, n = 11, nondiabetic rats treated with vehicle), control with H3S (CTRL, n = 12; nondiabetic rats treated with H3S), streptozotocin-induced diabetes group (STZV, n = 7; diabetic rats treated with vehicle), and streptozotocin-induced diabetes group (STZS, n = 9; diabetic rats treated with H3S). Minipumps were replaced at 2 wk. H3S or vehicle treatment lasted for 28 d. Blood glucose and blood H3S levels were measured with an Accu-Chek Advantage (Roche) and the amperometric H3S sensors.

**Statistical Analysis.** Data are expressed as means ± SEM. Statistical analysis was performed by ANOVA.

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Supporting Information

Fig. S1. Replacement of hydrogen sulfide (H$_2$S) attenuates cellular responses that lay downstream from hyperglycemic mitochondrial reactive oxygen species (ROS) production. (A) DNA strand breakage was measured in low (5.5 mM, LG) or high (40 mM, HG) glucose conditions at 7 d by using the Comet assay. High glucose induced an increase in DNA strand breakage compared with low glucose (*$P < 0.05$), and H$_2$S (300 $\mu$M) afforded a significant suppression of this response ($^#$P < 0.05). (Inset) Representative images are shown for the four respective groups (low/high glucose with and without 300 $\mu$M H$_2$S). (B) Activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) was measured by detection of the poly(ADP ribose) polymers by using Western blotting. High glucose induced an increase in PARP activation (*$P < 0.05$), and H$_2$S (300 $\mu$M) afforded a suppression of this response ($^#$P < 0.05). (Inset) Representative Western blot is shown for the four respective groups (low and high glucose with and without 300 $\mu$M H$_2$S) ($n = 5$).
Fig. S2. Acute administration of H₂S at the end of the hyperglycemic period does not affect mitochondrial oxidant production in endothelial cells placed in high extracellular glucose. Mitochondrial ROS production was measured in low (5.5 mM, LG) or high (40 mM, HG) glucose conditions at 7 d by using the MitoSOX red method, and H₂S (100–300 μM) was administered for 1 h at the end of the experiment. High glucose increased MitoSOX red oxidation (∗P < 0.05), but, when applied according to this protocol, H₂S failed to affect this response (n = 4).

Fig. S3. The ATP-sensitive potassium (K<sub>ATP</sub>) channel inhibitor glibenclamide does not prevent the protective effect of H₂S in endothelial cells placed in high extracellular glucose. Mitochondrial ROS production was measured in low (5.5 mM, LG) or high (40 mM, HG) glucose conditions at 7 d by using the MitoSOX red method in the presence or absence of H₂S (300 μM), with and without glibenclamide (10 μM, GLB) pretreatment. Glibenclamide slightly attenuated hyperglycemia-induced MitoSOX red oxidation but failed to influence the protective effect of H₂S on this response. ∗P < 0.05 indicates significant increases in MitoSOX red oxidation in high glucose compared with low glucose, and #P < 0.05 indicates significant suppression of this response by H₂S (n = 4).

Fig. S4. H₂S protects against the development of diabetic endothelial dysfunction in rat aortic rings placed in elevated extracellular glucose. Rat aortic rings were incubated in low (5.5 mM, LG) or high (40 mM, HG) glucose for 72 h. H₂S (200 μM) was administered every 8 h. High glucose induced a suppression of endothelium-dependent relaxant responses (∗P < 0.05), an effect that was prevented by H₂S (∗P < 0.05; n = 4).
Fig. S5. Streptozotocin-induced diabetes for 6 wk does not affect the expression of cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) in various tissues. Representative Western blots for CSE and CBS, loading control (actin), and densitometric analysis are shown in healthy control rats and in rats after 6 wk of streptozotocin-induced diabetes. Only CSE was detected in the thoracic aorta; CSE is more abundant than CBS in the heart, whereas only CBS was detected in the brain ($n = 6$).